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The relationship between non transferrin bound iron and iron overload in thalassaemia and sickle syndromes

Farrukh Tasnim Shah

MBBS; MRCP; MRCPATH

University College London

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University College London

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From work performed at the
Department of Haematology
University College London
98 Chenies Mews
London
WC1E 6HX

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MD abstract

The relationship between non transferrin bound iron and iron overload in thalassaemia and sickle syndromes

Iron overload is a major cause of morbidity and subsequent mortality in patients with thalassaemia major, its effects in thalassaemia intermedia and sickle cell anaemia are however less well known. The presence of non transferrin bound iron is well described in adult thalassaemia patients but it is unclear as to when NTBI appears and what relationship it has to ineffective erythropoiesis and end organ damage.

Data is presented on children with thalassaemia from a five-year prospective study showing that NTBI is present early in thalassaemia syndromes and this is probably due to ineffective erythropoiesis. In addition results from this study show that there is no relationship between markers of oxidative damage and NTBI in early childhood.

Following this a comparison of adult patients with sickle cell anaemia and thalassaemia is undertaken looking at NTBI and cardiac iron burdens assessed by MRI. The thalassaemia patients at high liver iron burdens have a significant risk of cardiac iron loading and when patients with sickle cell anaemia and thalassaemia major are matched for liver iron it is seen that cardiac iron loading is not seen in sickle patients and this may be because NTBI is lower in this group.

In the last chapter data is presented showing that serum pro-hepcidin is down regulated by NTBI, anaemia and erythropoietin in thalassaemia but not sickle syndromes. There is no clear relationship between pro-hepcidin and liver iron but hepcidin mRNA is down regulated by iron burden supporting the important role of this protein in iron regulation.

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This majority of the work presented in this thesis was performed at University College London and would not have been completed successfully without the aid of Dr Patricia Evans and Dr Roozina Rafique. They have been both excellent friends and colleagues and it has been an honour and pleasure to work with scientists of such high calibre. Dr Evans performed the NTBI assays presented in this thesis with some assistance from myself. Dr Evans performed the carbonyl assays and the transferrin saturations. Dr Rafique performed the MDA assays as well as the erythropoietin and CRP assays. Dr Rafique also helped with the RNA extractions. I would like to thank Andrew Laurie from the haemostasis lab for performing the soluble transferrin receptor assays.

Tim Diss from the department of histopathology taught me the method for RNA extraction and provided me with invaluable advice and guidance on RNA extraction from Paraffin blocks. I do not feel I would have been so successful at this without his help Dr Marco Novelli from the UCLH histopathology department provided the control liver blocks for the hepcidin RNA and helped ensure that sections were from areas not from areas affected by tumour. Professor Kaila Sria gave invaluable help on the PCR methodology and Monica Maracanthas and Nita Solanky at the Royal Free Medical School department of Biochemistry performed the PCR in his lab.

Our colleagues who are acknowledged specifically in the chapter collected all the data collection on the Sri Lankan and Canadian patients in the third chapter.

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I would like to dedicate this thesis to my family and my husband who are the foundation on which I stand; without their love and support this would all mean nothing.

Index of Contents

	Page
Title page	1
Abstract	2
Acknowledgements	3
Index of contents	5
Index of figures	13
Index of tables	15
List of abbreviations	17

Chapter One: introduction	20
---------------------------	----

The relationship between non transferrin bound iron and iron overload in thalassaemia and sickle syndromes

1.1 Introduction: The goals of the thesis	20
1.2 The Beta thalassaemias	23
1.2.1 Pathophysiology	23
1.2.2 Classification	24
1.2.3 Genetic basis of thalassaemia	24
1.2.4 Incidence and frequency of thalassaemia	25
1.2.5 Historical perspective	31
1.2.6 Transfusion iron overload in TM	35
1.2.7 Significance of iron overload in TM	37
1.2.8 Iron chelation therapy	38
1.2.9 Monitoring of TM	44

1.3 Thalassaemia Intermedia	47
1.3.1 Historical perspective	47
1.3.2 Molecular and genetic basis of thalassaemia intermedia	48
1.3.3 Complications associated with thalassaemia intermedia	49
1.3.4. Mechanisms of increased iron absorption in TI	50
1.4 The sickle syndromes	53
1.4.1 The genetic and molecular basis of sickle syndromes	53
1.4.2 Pathophysiology of sickle syndromes	54
1.4.3 incidence and frequency of sickle syndromes	55
1.4.4 historical perspectives	58
1.4.5 Complication of SCA that require transfusion	59
1.4.6 transfusion regimes in SCA	66
1.4.7 Iron metabolism and overload in SCA	67
1.5 Conclusions	68

Chapter Two	70
--------------------	-----------

Methods

2.1 Patients	70
2.1.1 Study sites	70
2.1.2 Patient cohorts	71
2.1.3 Ethics	71
2.2 Sample handling	71
2.2.1 Sample processing	72
2.2.2 Sample storage	72
2.3 Non transferrin bound iron	72
2.4 Transferrin saturation	74
2.5 Soluble transferrin receptor	77
2.6 Serum protein carbonyls	78
2.7 Malondialdehyde	79
2.8 Serum erythropoietin	80

2.9 C reactive protein	82
2.10 Serum pro-hepcidin	83
2.11 RNA extraction	85
2.12 Cardio magnetic resonance imaging	87
2.13 statistical analyses	88

Chapter Three

The relationship between markers of ineffective erythropoiesis, oxidative damage and non transferrin bound Iron in children with thalassaemia syndromes

3.1 Introduction	89
3.1.1 NTBI	91
3.1.2 Markers of oxidative damage	93
3.1.3 Markers of ineffective erythropoiesis	97
3.2 Study rationale	100
3.3 Patients and treatment centres	101
3.4 Methods	105
3.5 Statistical analysis	105
3.6 Overview of variables based on Underlying Disorder and Centres of treatment	106
3.6.1 Overview of results based on type of thalassaemia	106
3.6.2 Overview of differences in variables according to patient treatment Centres	108
3.6.3 Discussion of differences between patients at NTCK and UCLH/HS	112
3.7 The relationship between Ineffective erythropoiesis oxidative damage and NTBI	115
3.7.1 Factors affecting serum Erythropoietin	115

3.7.2 Variables affecting Transferrin receptors (sTfR)	121
3.7.3 Discussion	124
3.7.4 Variables affecting Malondialdehyde (MDA)	125
3.7.5 Variables affecting protein carbonyls	127
3.7.6 Discussion	128
3.8 Factors which affect NTBI	131
3.8.1 NTBI and its relationship with transferrin saturation and anaemia.	131
3.8.2 Relationship of NTBI to blood transfusion	133
3.8.3 Patients transfused less than 1000mls	133
3.8.4 NTBI in patients starting blood transfusions for the first time	135
3.8.5 Relationship of NTBI to ineffective erythropoiesis	138
3.8.6 discussion of factors affecting NTBI	141
3.9 Conclusions	143

Chapter Four	145
---------------------	------------

**Relationship of hepatic iron and NTBI to myocardial Iron and LV function;
contrasts between sickle and thalassaemia disorders**

4.1 Introduction	145
4.2 Techniques for estimating myocardial iron	148
4.2.1 Endomyocardial biopsy	148
4.2.2 Post mortem studies	149
4.2.3 MRI techniques for assessing myocardial iron	151
4.2.4 discussion	155
4.3 Aims and rationale of study	157
4.4 Patients	159
4.4.1 Patients for specific aim1	159

4.4.2 Patients for specific aim 2	159
4.4.3 Patients for specific aim 3	160
4.5 Methods	160
4.5.1 Cardiac T2*	161
4.5.2 Ejection fraction	162
4.5.3 Liver T2*	162
4.5.4 Statistical analysis	162
4.6 Factors affecting relationship between liver and myocardial T2* and heart function in thalassaemia	165
4.6.1 Cross sectional analysis of the relationships between liver and heart T2* and LVEF in TM and TI	165
4.6.2 Factors affecting change in cardiac T2* with time	171
4.7 Iron mediated morbidity, T2* and NTBI at matched ages and levels of iron loading.	180
4.7.1 Characteristics of the two cohorts	181
4.7.2 Complications in SCA and TM	183
4.7.3 Myocardial T2* in SCA and TM matched for LIC	184
4.7.4 Plasma NTBI and transferrin saturation in SCA and TM matched for LIC.	186
4.7.5 Relationship of NTBI to myocardial T2*	187
4.7.6 Relationship of liver iron to NTBI	188
4.7.7 Discussion and conclusions of comparisons of SCA and TM	189
4.8 Relationship between Cardiac T2* and Iron burden in TI	190
4.9 Conclusions	193

Chapter Five	196
---------------------	------------

**Hepcidin metabolism, iron overload and NTBI in thalassaemia
and sickle disorders**

5.1 Introduction	196
5.1.1 Structure, function and tissue expression of hepcidin	196
5.1.2 Effect of anaemia and hypoxia on hepcidin metabolism	198
5.1.3 Effect of inflammation on hepcidin metabolism	199
5.1.4 Relationship of iron overload to hepcidin in humans	200
5.1.5 Relationship of urine and liver hepcidin to plasma hepcidin	202
5.1.6 Relationship of serum hepcidin to serum pro-hepcidin	203
5.2 Aims and rationale	204
5.2.1 Patients for Aim 1	205
5.2.2 Patients for aim 2	206
5.3 Methods	208
5.3.1 Preparation of tissue for RNA extraction	208
5.3.2 PCR methodology	208
5.3.3 liver iron quantification	209
5.3.3 Prohepcidin ELISA	210
5.3.4 Liver iron measured by MRI	210
5.4 Results: Aim 1: the relationship between hepcidin and LIC in thalassaemia	211
5.4.1 Analysis of variables which may modulate hepcidin mRNA	212
5.4.2 Relationship of LIC to liver hepcidin mRNA	214
5.4.3 Relationship of Hepcidin mRNA to serum AST	217
5.4.4 Discussion: significance of hepcidin mRNA results	218
5.5 Results: Aim 2: pro-hepcidin, anaemia and iron overload in haemoglobinopathies	221
5.5.1 Variables affecting prohepcidin, independent of diagnosis	222
5.5.2 Relationship between pro-hepcidin and anaemia	226
5.5.3 Relationship between pro-hepcidin and LIC	228
5.5.4 Relationship between pro-hepcidin and NTBI	232
5.5.5 The relationship between pro-hepcidin and ALT	233
5.5.6 Discussion: Factors influencing serum prohepcidin levels	237
5.6 Conclusions	237

Chapter Six	238
Conclusions and discussion	

Bibliography	249
Appendix 1	271

Index of Figures

Chapter One		Page
	Figure 1.2.4.a: World distribution of Thalassaemia Syndromes and HbS.....	28
	Figure 1.2.4.b: Overall distribution of haemoglobin disorders (%carriers) by WHO region.....	29
	Figure 1.2.4.c: Geographical distribution of thalassaemia syndromes in England	30
	Figure 1.2.9.a: Under-transfused thalassaemia major patient.....	45
	Figure 1.2.9.b: Thalassaemic girl aged 17 years.....	46
	Figure 1.4.3.a: Distribution of sickle cell anaemia in the old world.....	56
	Figure 1.4.3b: Geographical distribution of sickle cell disease in England.....	57
Chapter Two		
	Figure 2.4.2.a: Transferrin saturation gel electrophoresis.....	76
Chapter Three		
	Figure 3.7.1.a: Haemoglobin versus erythropoietin in all patients.....	116
	Figure 3.7.1.b: Relationship of Log Erythropoietin (Ln EPO) to Haemoglobin in all patients	117
	Figure 3.7.1.c: Relationship between EPO and sTfR in all patients.....	119
	Figure 3.7.2.a: Relationship of sTfR to haemoglobin.....	122

Figure 3.7.2.b: sTfR and serum ferritin in all patients.....	123
Figure 3.8.1.a: Transferrin saturation and NTBI for all patients.....	132
Figure 3.8.3.a: Transferrin saturation for 15 patients transfused less than 1000ml of blood	134
Figure 3.8.4 a: NTBI pre-transfusion and post transfusion in..... those patients undergoing their first ever transfusion	136
Figure 3.8.4 b: NTBI and cumulative iron load in patients with TM..... who had their first transfusion as part of this study	137
Figure 3.8.5.a: Relationship of sTfR to NTBI in UCLH/HSC patients..... who had received less than 1g of iron from blood transfusion	140
Figure 3.8.5.b: sTfR and NTBI in TI and TM patients.....	141

Chapter Four

Figure 4.2.2.a: Cardiac and LIC correlations from pre-chelation era.....	150
Figure 4.6.1.a: Relationship between EF and cardiac T2*	165
Figure 4.6.1.b: The relationship between LVEF and cardiac T2* in TI and TM	167
Figure 4.6.1.c: The relationship between LVEF and R2*..... in TI and TM	168
Figure 4.6.1.d: The relationship between LIC to LVEF in..... TI and TM patients	169
Figure 4.6.1.e: The relationship between 1/T2* and LIC.....	170
Figure 4.6.2.a: T2* of liver at T0 and then 48 hourly intervals..... post DFO infusion	172
Figure 4.6.2.b: Cardiac T2* at T0 and then up to 10 day..... post DFO infusion	173

Figure 4.6.2.c: Changes in cardiac T2* over time in.....	176
patients 1 to 13	
Figure 4.6.2.d: Patient 4 treated with intensive chelation.....	177
Figure 4.6.2.e: The relationship between liver iron and change.....	179
in myocardial T2*	
Figure 4.7.3.a: Cardiac T2* in SCA and TM patients matched.....	184
for iron loading	
Figure 4.7.5.a: Relationship of NTBI to myocardial T2*.....	187
Figure 4.7.6.a: Relationship of LIC to NTBI in patients matched.....	188
for liver iron	

Chapter Five

Figure 5.4.2.a: LIC and hepcidin mRNA for all patients.....	214
Figure 5.4.2.b: Liver iron and Hepcidin mRNA, excluding.....	215
HCV RNA positive	
Figure 5.4.2.c: Hepcidin mRNA subdivided according to LIC.....	216
Figure 5.4.3.a: Relationship of Hepcidin mRNA to serum AST.....	217
Figure 5.5.2.a: Relationship of pro-hepcidin and Hb in all patients.....	226
Figure 5.5.2.b: Relationship between pro-hepcidin and Hb	227
Figure 5.5.3.a: Prohepcidin and LIC in patients.....	229
Figure 5.5.3.b: pro-hepcidin in 3 cohorts matched for LIC.....	230
Figure 5.5.4.a: NTBI and serum pro-hepcidin in all patients with.....	233
detectable NTBI	

Index of Tables

Chapter 3	Page
Table 3.3.1.1: Patient characteristics for all groups.....	104
Table 3.6.1.1: Mean values for all parameters in all patients.....	107
Table 3.6.2.1: Table of parameters for all UCLH/HSC.....	109
treated patients	
Table 3.6.2.2: parameters in all NTCK treated patients.....	111
Table 3.8.4.1: NTBI pre-transfusion and post transfusion in.....	135
those patients undergoing their first ever transfusion	
 Chapter 4	
Table 4.6.1.1: Baseline characteristics of the study population.....	166
Table 4.6.2.2: Change in cardiac T2* with chelation treatment over time.....	175
Table 4.6.2.3: The relationship between LIC and cardiac T2*.....	180
Table 4.7.1.1: Baseline characteristics of the two cohorts.....	181
Table 4.7.2.1: Complications found in SCA and TM cohorts.....	182
Table 4.7.2.1: LIC, cardiac T2*, NTBI and transferrin saturation.....	183
for all patients	
Table 4.8.1.1: Patient characteristics with thalassaemia intermedia.....	191
Table 4.8.1.2: Complications in TI patients.....	191
Table 4.8.1.3: LIC, cardiac T2*, LVEF and NTBI (TI).....	193

Chapter 5

Table 5.2.1.1: Patient characteristics for aim 1.....	206
Table 5.2.2.1: Patient Characteristics for aim 2.....	207
Table 5.4.1.1: Hepcidin mRNA, ferritin and LIC in patients.....	212
Table 5.4.1.2: Analysis of factors which may affect liver.....	213
hepcidin mRNA levels	
Table 5.5.1.1: Variables associated with prohepcidin levels.....	223
in all patients	
Table 5.5.1.2: Results (mean) for all patients according to.....	225
type of anaemia	
Table 5.5.3.1 Thalassaemia Intermedia, Major and SCA.....	231
patients matched for liver iron	

List of abbreviations

AA	amino acid
ALA	delta aminolaevulinic acid
BFU	burst forming unit
BHT	butylated hydroxytoluene
CFU	colony forming unit
CMR	cardio magnetic resonance
CRP	c reactive protein
DCT1	divalent cation transporter 1
Dcyt b	duodenal Cytochrome b
DEXA	dual energy x-ray absorptiometry
DFO	desferrioxamine
DNA	deoxyribonucleic acid
DNP	dinitrophenylhydrazine
ECHO	echocardiography
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
EPO-R	erythropoietin receptor
GC MS	Gas Chromatography- Mass Spectroscopy
Hb	haemoglobin
HCL	hydrochloric acid
HEPC1	hepcidin gene
HFE	haemochromatosis gene
HH	hereditary haemochromatosis
HH	hereditary haemochromatosis
HPFH	hereditary persistence of fetal haemoglobin
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSC	hospital for sick children Toronto Canada

IL	interleukin
IV	intravenous
IVS	intervening sequence
JAK 2	Janus Kinase 2
kDA	kilo Dalton
LCR	locus control region
LIC	liver iron concentration
LnEPO	log EPO
LPS	lipopolysaccharide
LVEF	left ventricular ejection fraction
MDA	malondialdehyde
MRI	magnetic resonance imaging
mRNA	messenger RNA
MTR	magnetic transfer ratio
NO	nitric oxide
NOD-SCID	non obese diabetic-severe combined immunodeficiency
NTA	nitritotriacetate
NTBI	non transferrin bound iron
NTCK	national thalassaemia centre Kurunegala Sri Lanka
PCV	packed cell volume
QTL	quantitative trait locus
RE	reticuloendothelial
RNA	ribonucleic acid
ROS	reactive oxygen species
SC	subcutaneous
SCA	sickle cell anaemia
SCF	stem cell factor
SEM	standard error of the mean
SIR	signal intensity ratio
SNP	single nucleotide polymorphism
sTfR	soluble transferrin receptor
TBA	thiobarbituric acid
TBARS	TBA reactive substances
TCA	trichloroacetic acid
TI	thalassaemia intermedia

TM	thalassaemia major
TNF	tumour necrosis factor
TRAP	total peroxy radical-trapping antioxidant parameter
tRNA	transfer RNA
UCLH	university college london hospitals UK
uhhepcidin	urinary hepcidin
USF2	upstream stimulating factor 2
UTR	untranslated region
VCAM	vascular adhesion molecule 1
VEGF	vascular endothelial growth factor
WBC	white blood count
WHO	world health organization

Chapter One

The relationship between non transferrin bound iron and iron overload in thalassaemia and sickle syndromes

1.1 Introduction: the goals of this thesis

The purpose of the work presented in this thesis is to examine the relationship between plasma non-transferrin bound iron (NTBI), iron overload and iron distribution to organs in patients with thalassaemia and sickle cell syndromes.

In thalassaemia syndromes, iron overload may result from increased gastrointestinal iron absorption or from repeated blood transfusions. Patients with thalassaemia intermedia (TI) may slowly develop iron overload secondary to increased gastrointestinal iron absorption, even without receiving blood transfusions. In thalassaemia major (TM), iron accumulation occurs more rapidly as a result of repeated transfusions from an early age. If inadequately chelated, these patients will develop endocrinopathies and cardiac failure from iron deposition in these organs. With the early introduction of chelation therapy, these consequences are now less commonly seen in TM than previously (Borgna-Pignatti, *et al* 2004), although patients may still suffer serious complications from iron overload such as endocrinopathies and cardiac decompensation if inadequately treated (Borgna-Pignatti, *et al* 1998, Borgna-Pignatti, *et*

al 2004, Zurlo, *et al* 1989). The extent to which critical threshold levels of total body iron (Olivieri and Brittenham 1997) or the duration of exposure to high iron burdens (Gabutti and Piga 1996) or the distribution of iron to different organs contribute to the net effects of transfusional iron overload remain unclear. Up until very recently, evidence of iron overload was based on tests such as serum ferritin, liver iron content by biopsy, or on the effect of iron overload on organ function such as endocrine function, glucose tolerance tests and left ventricular function. However with the recent use of Magnetic Resonance Imaging (MRI) it has been possible to examine iron distribution and the relationship between myocardial and hepatic iron loading. The factors that determine iron loading into the heart and endocrine organs in TM remain unclear. A key candidate that may be critical in determining the distribution of iron to various organs is plasma NTBI. However there have been no studies that examine the relationship between the progression of iron overload and NTBI levels in thalassaemia or sickle cell disorders. In this thesis, a longitudinal assessment is undertaken in thalassaemia major to see if a critical iron burden exists at which plasma NTBI appears.

In sickle cell anaemia (SCA), the relationship between iron overload and distribution to organs such as the heart is even less well defined than in TM. In SCA ineffective erythropoiesis does not play a major role and, in the absence of blood transfusion, iron overload is not seen. However, the management of SCA in recent years has increasingly included the use of blood transfusions to prevent and treat complications such as stroke, chest syndrome and venous ulcers. This in turn has resulted in patients developing significant iron overload, necessitating treatment of some patients with iron chelating agents. End organ damage from iron overload is less well described in SCA than in TM, even in the context of a high transfusional iron load and high hepatic iron loading. It has been suggested that iron may be dealt with differently in SCA with more sequestering of iron in the reticuloendothelial system (Walter, *et al*

2006). In order to examine this possibility in the thesis, multi-transfused SCA patients are matched to thalassaemia patients for liver iron concentration, and relative levels of cardiac iron deposition as well as relative NTBI levels compared between these patient groups.

The recent discovery of molecules involved in iron regulation such hepcidin have provided insight into how both gastrointestinal iron absorption and iron release from macrophages is regulated. Theoretically levels of hepcidin could impact on NTBI levels and hence on secondary iron distribution by NTBI to various organs in transfusional iron overload. The relationship between hepcidin, iron overload and NTBI in patients with thalassaemia and SCA has not been compared previously.

Specific questions to be addressed in this thesis are:

- 1) The age at which NTBI appears in children with thalassaemia syndromes, and whether it is present early or develops later in relation to iron overload (chapter 3)
- 2) The relationship between NTBI and markers of ineffective erythropoiesis and their relationship with markers of oxidative damage (chapter 3):
- 3) A comparison of the relationship between NTBI and iron loading in the liver and myocardium in thalassaemia syndromes and sickle cell anaemia (chapter 4)
- 4) The relationship between hepcidin, iron overload and NTBI in patients with thalassaemia and SCA (chapter 5).

Before addressing these specific questions, a brief description of thalassaemia and sickle syndromes is given with particular reference to the contrasting ways in which iron overload may develop in thalassaemia major, thalassaemia intermedia and sickle cell disease.

1.2 Beta Thalassaemia Syndromes

The thalassaemia syndromes comprise a heterogeneous family of disorders that arise from defects in the rate of synthesis of alpha or beta chains that form the globin component of haemoglobin.

1.2.1 Pathophysiology

Beta thalassaemia occurs due to reduced or absent synthesis of β chains. This results in an excess production of α chains, which cannot form tetramers and therefore precipitate in the developing erythroid cell forming large intracellular inclusion bodies. These excess chains are very unstable and become associated with the red cell membrane and ultimately result in the destruction of the developing erythroid precursors in the intra-medullary space by a number of mechanisms including oxidative damage to cell membranes and interference with cell division (Rund and Rachmilewitz 1995, Shinar and Rachmilewitz 1990).

This premature destruction of erythroid precursors results in the development of anaemia due to ineffective erythropoiesis. Those cells that do mature and enter the circulation are prematurely destroyed due to the presence of inclusion bodies in the splenic microcirculation. Hence the anaemia in thalassaemia syndromes is due to both ineffective erythropoiesis and a shortened red cell survival.

Variant β haemoglobins such as HbE (β^{26} GAG-AAG, glutamic acid –lysine), when co-inherited with the β thalassaemia mutations can result in thalassaemia syndromes of varying severity (Rees, *et al* 1998).

1.2.2 Classification

Thalassaemia syndromes can be subdivided according to their genetic causation or according to their phenotypic presentation. Normal globin production results from balanced synthesis of alpha globin from duplicated genes on chromosome 16 and single β genes on chromosome 11. The description below will focus mainly on β globin thalassaemia resulting from reduced or absent β globin synthesis, as it is β thalassaemia that is studied in subsequent chapters.

Classification according to phenotype

The most commonly used and simplest classification is according to the clinical severity of the anaemia. This results in 3 main subdivisions.

- Carriers (single β mutation) of the defect with asymptomatic anaemia (Thalassaemia trait)
- Severe transfusion dependant anaemia (Thalassaemia Major; TM): usually homozygote or compound heterozygote for β mutations
- Intermediate severity of anaemia that may or may not require transfusion support. (Thalassaemia Intermedia, TI) : genetically heterogeneous (see below)

Classification of β thalassaemias according to genotype

β thalassaemia mutation can be classified according to amount of globin chain that is produced. With some mutations no globin is synthesised and is termed β^0 thalassaemia. In others the globin chain is synthesised but in reduced amounts and is known as β^+ thalassaemia. The majority of the β thalassaemias tend to be due to point mutations rather than deletions. However, because δ and the duplicated γ genes are upstream of the β gene, large deletional mutation of the β gene may also involve deletions of these genes (e.g. $\delta\beta$ thalassaemias). The $\delta\beta$ Thalassaemia, $\epsilon\gamma\delta\beta$ Thalassaemia, δ Thalassaemia, and Hereditary Persistence of Fetal Haemoglobin (HPFH) will not be discussed in this thesis as individual disorders but will be discussed in so far as their role as modifiers of the thalassaemia phenotype. β like thalassaemia syndromes may also result from a compound heterozygous state of a β mutation with a β haemoglobin variant that is present in reduced quantities such as E β thalassaemia syndromes.

1.2.3 Genetic basis of β thalassaemias

Over a 1000 mutations affecting the globin genes have been identified to date (Clark and Thein 2004) with about 200 of these resulting in β thalassaemia syndromes (Flint, *et al* 1998) of varying clinical severity. A variety of mutations can occur in the globin genes varying from simple point mutations to large deletions, insertions or alterations that result from more than one mutation. The site at which a mutation occurs will also affect whether there is an abnormal globin chain, reduced amounts of a normal globin chain or no globin chain synthesis. Mutations at one of the promoter sites

invariably result in reduced production of globin whereas mutations occurring at initiation sites will lead to no globin chain production. Splice junction mutations (donor or acceptor sites) will result in absent globin production due to abolition of normal splicing. Whereas mutations in the consensus splice sites causes reduced normal splicing but will result in some globin chain synthesis. Mutations at poly A sites lead by and large to reduced production of globin chains. Mutations can occur outside the globin genes leaving the genes themselves intact and result in reduced globin synthesis such as deletions of the LCR of the globin genes resulting in $\epsilon\gamma\delta\beta^0$ thalassaemia, or deletion of the α globin enhancer site, which is at the 3' end of the α gene.

1.2.3.1 Genetic modifiers of the phenotype

The thalassaemia syndromes are clinically diverse disorders: genetic modifiers are known to exist that account for some of the variability seen within any given genotype. There are however very complex interactions between environmental and other genetic factors that may affect the phenotype of the thalassaemia and much work still needs to be done to identify these factors. Certain factors are well known to affect the phenotype of the disorder. The primary modifier of the genotype is the site of the globin chain defect itself. The secondary modifiers are due to variation in the production of HbF, the alpha globin defect and the coinheritance of a β globin chain variant. As survival in patients has improved more subtle modifiers of the phenotype have been identified and are described as tertiary modifiers (Thein 2004). Infection with malarial parasites in worsening the severity of anaemia particularly in the younger patients can be considered a tertiary modifier of the phenotype (Premawardhena, et al 2005). Co-inheritance of the mutation associated with Gilberts syndrome leads to a higher propensity to develop gallstones (Bosma, et al 1995) and can be considered a tertiary

modifier. The coinheritance of HFE defects in particular the C282Y in thalassaemia intermedia is a tertiary modifier of iron loading (Rees, *et al* 1997).

Osteoporosis is extremely common in thalassaemia syndromes and may be due to a combination of factors but workers have recognised associations with the vitamin D receptor (VDR)(Dresner Pollack, *et al* 2000), and Collagen type 1a (COL1A1) gene (Perrotta, *et al* 2000). In this thesis (chapter 5) the role of hepcidin, which is a negative regulator of iron absorption, is explored and its role in modifying iron distribution and hence iron mediated damage in thalassaemia and sickle syndromes.

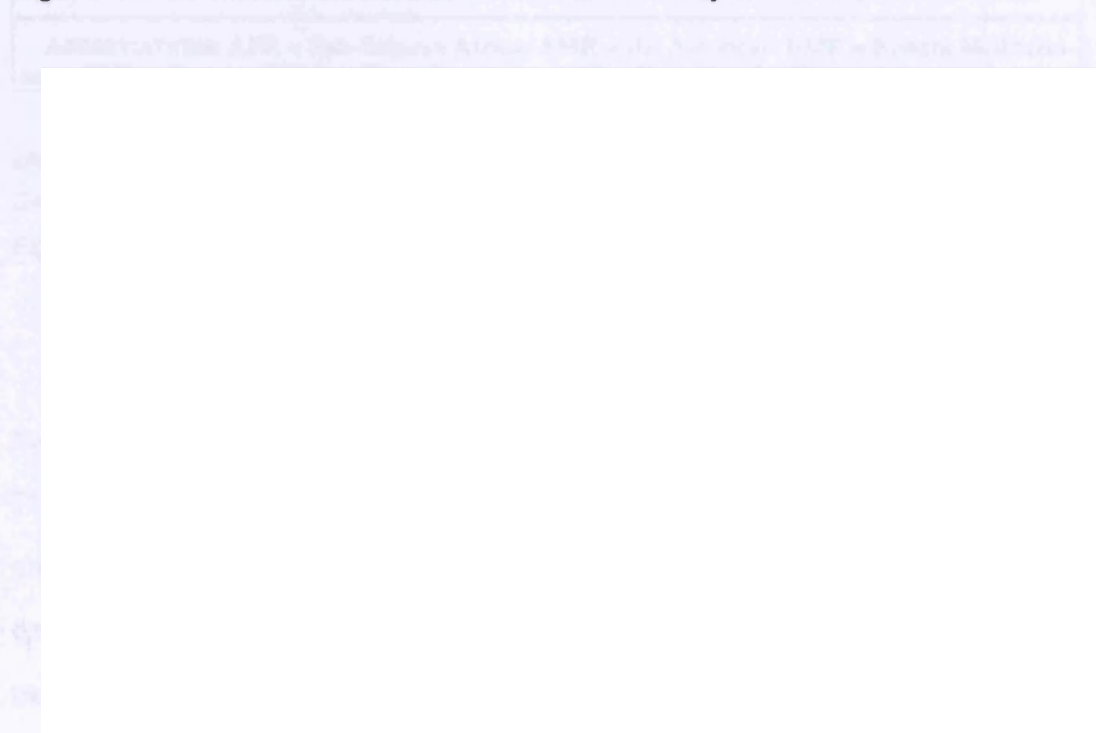
1.2.4 Incidence and Frequency of Thalassaemia

The frequency of the alpha and beta globin defects have not been analysed in all countries worldwide, primarily due to lack of resources and greater clinical priorities. However a number of workers have undertaken these studies in individual countries. World wide figures from the WHO suggest that there are 270 million carriers for globin gene defects and that 300,000 to 400,000 children are born annually with a severe haemoglobin disorder (Weatherall and Clegg 2001). As the socioeconomic circumstances improve world wide a greater number of children will survive into adulthood and the economic burden of treatment of the haemoglobinopathies will increase manifold.

Micro-mapping by individual groups have shown how the frequency of thalassaemia or variant haemoglobins such as HbE can vary significantly throughout a region often just a few miles apart (Ahmed, *et al* 2002, Araujo, *et al* 2003, Old, *et al* 2001, Thong, *et al* 2005). This may be due to social factors as in many south East Asian communities, marriages occur within tribal/family group confines and this has resulted in a higher gene frequency in specific groups e.g. in Pakistan the frequency of

thalassaemia trait is 5.4% for the general population (Khattak and Saleem 1992) but on family testing based on index cases the incidence was 31% and there was a 25% risk that the carrier would be in a marriage at risk of producing an affected child (Ahmed, *et al* 2002); in Oman the carrier rate for the indigenous Arab population is 1-2% (Daar, *et al* 1998) but in the Belushi tribe which migrated from the province of Baluchistan (Pakistan) the incidence is 10% (Angastiniotis and Modell 1998)

Figure 1.2.4.a World distribution of Thalassaemia Syndromes and HbS



Legend: world distribution of Haemoglobinopathies from Christianson, A. et al. BMJ 2004;329:1115-1117. credited to the WHO GENOMIC RESOURCE CENTRE

Figure1.2.4.b Overall distribution of haemoglobin disorders (%carriers) by WHO region

Legend: WEATHERALL, DAVID J. *Keynote Address: The Challenge of Thalassemia for the Developing Countries. Annals of the New York Academy of Sciences* **1054** (1Cooley's Anemia: Eighth Symposium), 11-17. doi: 10.1196/annals.1345.002

Due to migration the prevalence of the TM and TI has altered in a number of countries. In the UK there are approximately 800 patients with TM (Modell, *et al* 2000). The greatest increase in births of thalassaemic children is in the Asian communities and predominantly Pakistani families (Modell, *et al* 2000). The UK distribution of these disorders reflects this where large pockets of ethnic minorities settled the thalassaemia frequency is greatest (Hickman, *et al* 1999).

Figure 1.2.2.4.c Geographical distribution of thalassaemia major and intermedia in England



Legend: reproduced from (Hickman, et al 1999) Map of England showing the majority of TM and TI births are in London, the Midlands and the north of England. These are the traditional areas where migrant families settled.

1.2.5 A Historical Perspective

The initial description of thalassaemia by Thomas Cooley in 1925 (Cooley and Lee 1925) was of 5 children who presented with anaemia, hepatosplenomegaly, discolouration of the skin and classical facies. They all had a leukocytosis which was not of the leukaemic type but predominantly normoblasts and in 2 patients many reticulated cells. There was normal or increased resistance to osmotic lysis of red blood cells from these to haemolysis by hypotonic solutions. In addition all these children had a classical mongoloid facies.

Further publications both in Europe and in the USA defined the complications of anaemia in TM. These children usually presented within the first year of life and rarely survived beyond the first few years of life. Profound anaemia, failure to thrive, recurrent infections, and progressive abdominal distension due to hepatosplenomegaly were uniformly described. Those with the severest anaemia would die within the first few months of life from cardiac failure and /or infection and those with less severe anaemia would start to develop other manifestations of the anaemia such as poor growth and venous ulceration. Thrombocytopenia and neutropenia secondary to hypersplenism along with associated infective and bleeding complications was also seen.

X ray images of the bones would show cortical thinning, and a 'moth eaten' or lace like' appearance, of the intramedullary space in the small bones. The skull X rays were the most dramatic with the classical 'hair on end' appearance due to expansion of the diploic space. Pathological fractures were frequent occurrences. Extramedullary haematopoiesis could be seen on chest X rays and some children would go on to develop spinal cord paraparesis due to cord compression. As children developed beyond the age of 7 years there was evidence of delayed growth and sexual development that was thought to be secondary to severe anaemia and possibly the siderosis from increased gastrointestinal iron absorption (Weatherall and Clegg 1972).

The prognosis of children with TM was poor with one of the best descriptions in Man's Haemoglobins by Lehmann and Huntsman (Lehmann and Huntsman 1966) where in chapter 15 the attitude to the treatment was described as:

"Somewhat similar to the attitude of the physician treating children with leukaemia".

It was stated that: *"it was possible to nurse these children through to the age of 10 or 11"* and *"the condition is so hopeless that attention must be given to the parents and family "*.

However, advances were being made in the care of thalassaemic children primarily aimed at improving quality of life and included splenectomy and transfusion both of which had significant impact on the outcome.

1.2.5.1 History of blood transfusion use

Blood transfusion as a therapeutic measure has only been a practical option since the late 1920s as the ability to use blood group typing became more widespread. Blood transfusion was used as early as 1665 when transfusions were carried out between dogs to confirm that transfusion was safe. Mollison (Mollison 1979) described the first blood transfusion to a human that was published by Denis in 1667.

" When a man who had fallen into a phrensy occasioned by a disgrace he received" was transfused calf's blood.

"It was hoped that by its mildness and freshness it would allay the heat and ebullition of his Bloud"

Over the next few years more animal to human transfusions occurred and the outcome was so poor that they were banned by the Paris society of Physicians in 1678. James Blundell first performed successful human-to-human transfusions in 1818 and during the ensuing years he performed 10 transfusions 5 of which were successful. (<http://www.bloodbook.com/index.html>).

No real advances occurred in transfusion medicine until 1901 when Karl Landsteiner first described agglutination reactions on mixing different blood samples and concluded only 2 antigens were needed to explain the 4 groups of people. It was not until 1908 that the benefits of cross matching for safe transfusion practice were observed and the first matched blood transfusion performed (Race and Sanger 1975) . Over the next 10 years anticoagulants were developed which allowed longer storage of blood and the British established the first 'blood depot' during World War I. The British Red Cross instituted the first blood transfusion service in the world in 1926 around the same time as the first description of Cooley's anaemia.

1.2.5.2 Impact of blood transfusion in thalassaemia management

As early as 1925 in Cooley's original paper one of the children was given transfusion:

"The only results in treatment have been with a mixture of spleen and red bone marrow combined with administration of hydrochloric acid. One transfusion caused only slight transient blood change, and urine examination showed the transfused blood underwent rapid haemolysis. A more recent transfusion was followed by a better blood picture and less haemolysis"

The use of blood was dangerous and difficult and it was only by the late 1930's that blood transfusions were being used to treat the anaemia in some patients, albeit at infrequent intervals, resulting in children surviving longer but still having the majority of the manifestations of severe anaemia. Wintrobe's view on transfusion therapy in 1946 (Wintrobe 1946) was rather pessimistic stating that

"transfusion is of temporary value".

It was not until 1964 when Wolman published his data (Wolman 1964) on maintaining children with a high mean haemoglobin that the evidence for improved survival became clear. The children in Wolman's study had fewer complications from anaemia, better growth and quality of life in the first decade. Over the next few years an increasing body of evidence was accumulated supporting a high transfusion regime which allowed remarkably good growth, development and health throughout childhood (Beard, *et al* 1969, Piomelli, *et al* 1969). It was noted that children on high transfusion regime had less splenic and hepatic enlargement and the cardiomegaly was less marked (Piomelli, *et al* 1974). Based on the evidence from these and other studies it was concluded that high transfusion regimes were desirable in order to maintain good quality of life. Weatherall and Clegg (Weatherall and Clegg 1972) in Chapter 9 (Management of Thalassaemia) recommended that a pre-transfusion Hb of 10g/dl be maintained at all times to ensure a good quality of life with a judiciously timed and well followed up splenectomy if necessary, accepting that:

'At the moment there seems little doubt that iron overload will kill children on any type of transfusion regime'

Deaths were now no longer occurring in the first decade from severe anaemia but increasingly seen in the 2nd decade of life from complications secondary to iron overload in the myocardium, in particular sudden cardiac death due to dysrhythmia and cardiac failure (Engle 1964). The increasing appearance of endocrinopathies and diabetes from iron deposition in other vital tissues (Weatherall and Clegg 1972, Wolman and Ortolani 1969) was also becoming more recognised.

1.2.6 Transfusional iron overload in thalassaemia major

1.2.6.1 Rate of iron loading from blood transfusion

Iron in the average non iron overloaded healthy adult is present to the order of around 40-50mg/kg body weight. Most of this is present in the form of haemoglobin (30mg/kg) with the remainder divided between myoglobin (4mg/kg), iron containing enzymes (2mg/kg), and storage iron in the form of ferritin and haemosiderin. Storage iron tends to be between 0 -2000mg and is primarily found in the liver, spleen and bone marrow. Dietary iron absorption is approximately 1-2mg per day and this is matched by the iron lost through exfoliation of gut mucosal cells, skin cells and those from the urinary tract. In women additional iron is lost through endometrial shedding. There is no excretory mechanism for iron, and there is only a limited mechanism for modulating iron absorption (Conrad, *et al* 1999).

Iron may accumulate as a result of increased iron absorption thorough the gastrointestinal tract or may be iatrogenic via blood transfusions. In the thalassaemia syndromes iron overload occurs due to blood transfusions and to some degree secondary to increased GI iron absorption in TM and predominantly via GI iron absorption in the TI. A unit of red cells, processed from 420 mL of donor blood, contains approximately 200 mg of iron (0.47 mg iron/mL of whole donor blood or 1.16 mg iron/mL of pure red cells). In splenectomised TM patients, in order to maintain an average Hb of 12g/dl, they will receive an average of 300 mL of whole blood per kg per annum (range 200-400 mL whole blood/kg) (Modell 1976). This will equate to approximately 28 mg of

iron in an adult weighing 70 kg. In non-splenectomised TM patients the transfusion requirements are generally higher and this may lead to a greater degree of iron loading, however the use of blood transfusion regimens designed to maintain a high mean Hb regimen may reduce the extent to which the spleen contributes to an increased blood transfusion requirement (Modell 1976). The increased GI iron absorption results in for a further 1-4 mg daily (Pippard and Weatherall 1984), therefore iron will accumulate at a rate of 0.4-0.5 mg/kg/day or 28-35 mg/day in a splenectomized adult patient with TM.

1.2.6.2 Clinical effects of transfusional iron overload

The first evidence of severe iron overload was often the failure of the child to go into puberty or to complete pubertal development (Modell 1976). The reason for this delayed pubertal growth spurt was unclear and growth hormone deficiency was excluded as a cause (McIntosh 1976, Zaino, *et al* 1969). It was not until Landau who found reduced gonadotrophins (Landau, *et al* 1978) and a reduced response to LHRH that this axis was considered as a cause for the delayed puberty. Around the same time the incidence of other endocrinopathies such as hypothyroidism and hypoparathyroidism were documented (Flynn, *et al* 1976, Lassman, *et al* 1974). Diabetes was a well recognised complication and tended to occur in children with severe iron overload over the age of 10 years (Toccafondi, *et al* 1970). Cardiac iron loading occurred mostly in adolescence in the pre-chelation era. The most serious and frequently fatal consequence of transfusional siderosis was cardiac failure or dysrhythmia due to iron deposition in the myocardium. Those patients who did not die of cardiac failure would by late adolescence have developed hepatic cirrhosis. As a result of the advances made in transfusion support most patients were surviving into adolescence and then dying of complications secondary to iron overload.

1.2.6.3 Mechanisms of toxicity of iron overload

Iron that is not bound to transferrin or stored within ferritin, causes oxidative damage by free radical generation by the Haber- Weiss reaction (Halliwell and Gutteridge 1990, Olivieri and Brittenham 1997). Hydroxyl radicals (OH^\bullet) that are released as a consequence of this reaction can then cause lipid peroxidation, oxidation of cellular proteins, lipoproteins, nucleic acids, and other cellular constituents (Aust, *et al* 1985, Halliwell and Gutteridge 1990). Any cell death that occurs will result in fibrosis and loss of organ function and hence the complications of iron overload develop (Aust, *et al* 1985, Britton, *et al* 2002, Fleming and Bacon 2005, Houglum, *et al* 1990, Junge, *et al* 2001). Oxidative damage is thought to be the key reason for the fibrosis and cell death in affected organs such as the liver in thalassaemic patients.

It is however, highly likely that a critical iron burden is necessary before damage occurs (Olivieri and Brittenham 1997) and quite probable that duration of exposure to high iron burdens is also important (Gabutti and Piga 1996). This is probably the reason why children developed diabetes around the age of 10 years in the pre chelation era and cardiac failure during adolescence. Modern management has resulted in a dramatic decline in iron overload associated complications. The primary reason for this decline has been the use of chelation regimes to remove the excess iron.

1.2.7 Significance of Non transferrin bound iron in iron overload

Iron exists in human sera as transferrin bound iron, however once transferrin becomes fully saturated then free iron is found in the form of non-transferrin bound iron

(NTBI). NTBI was first identified by Hershko in thalassaemic patients in whose sera chelatable iron was measured in the presence of a fully saturated transferrin and when thalassaemic sera was added to normal sera the chelatable iron could no longer be detected (Hershko, *et al* 1978). NTBI was then found to occur in other iron loading conditions such as haemochromatosis and was linked to oxidative damage (Gutteridge, *et al* 1985).

To date the exact nature of NTBI is undefined and it is thought to be composed of a mixture of oligomeric and monomeric complexes of iron with ligands such as citrate, phosphate and albumin (Hider 2002). It is probable that the nature of the NTBI varies according to the type and degree of iron overload (Breuer, *et al* 2000). In addition it is known that in some iron overload states, patients may have NTBI in the presence of unsaturated transferrin (Gosriwatana, *et al* 1999). It is thought that this type of NTBI is inaccessible to transferrin and some NTBI species can cause oxidative damage by free radical generation by the Haber- Weiss reaction (Halliwell and Gutteridge 1990, Olivieri and Brittenham 1997). In this thesis the relationship between NTBI and markers of oxidative damage (malondialdehyde and protein carbonyls) in children who have TM or TI will be presented in Chapter 3.

1.2.8 Iron chelation therapy

By the late 1960's there was a desperate need for an effective, safe and acceptable method for iron removal / chelation. Weatherall and Clegg (Weatherall and Clegg 1972) gave a comprehensive review on the status of chelating agents in 1972 which was relatively pessimistic about the potential benefits:

"It takes at least 5 to 10 years to assess any regime designed to reduce mortality from iron overload and few physicians have been keen to examine critically a prolonged regime which is expensive, painful and probably of marginal benefit"

Work on chelation therapy had been ongoing since the early 1960's, and the development of safe and effective chelation therapy was to prove just as important as appropriate blood transfusion support in modifying the natural history of thalassaemia.

1.2.8.1 Desferrioxamine

Desferrioxamine (DFO) was first introduced in the early 1960's as a chelating agent to treat acute iron poisoning (Moeschlin and Schnider 1964, Pittman 1964). It belongs to the hydroxamic acid class of chelators and is a hexadentate chelator, able to bind 1 ferric iron per molecule. It binds ferric iron with very high affinity producing ferrioxamine, which is excreted both in the urine (30-50%) and faeces (Bianco, *et al* 1984, Herskho and Rachmilewitz 1978, Pippard 1989, Pippard, *et al* 1982, Walsh, *et al* 1964) It has a relatively short half-life of 5 to 10 minutes in the plasma (Summers, *et al* 1988) and it was noted by Sephton-Smith that maximal excretion of iron occurred when it was administered as a slow IV infusion (Smith 1962). However there was concern about loss of efficacy over time and the potential for achieving a net negative iron balance (Modell 1979). A review on chelation therapy in 1969 (Waxman and Brown 1969) was very downbeat about the benefit of chelator therapy.

It was only when Barry (Barry, *et al* 1974) published data from a controlled trial on iron chelation that the benefits of iron chelation became clear. The study compared patients on 6 days a week treatment with 500mg IM DFO and 2G IV with transfusion, to a control arm who received no DFO. A significantly reduced iron burden as measured by

liver iron and ferritin and the lesser degree of hepatic fibrosis was shown in those patients who received iron chelation. Follow-up data from this study in 1981 showed that of the 9 patients in the treatment arm only 1 had died whereas 6/10 in the control arm had died. This is the only placebo-controlled trial on a chelating agent that has been published and these results changed significantly the treatment of patients with thalassaemia. The short half-life and greater excretion of iron seen with infusions prompted a number of workers to assess the efficacy of SC DFO as a treatment option. A number of studies were published in 1978 and 1979, which showed considerable reductions in ferritin, and liver iron and improvement in liver function tests (Hoffbrand, *et al* 1979, Weiner, *et al* 1978). Pippard (Pippard, *et al* 1978b) showed the potential for chelation therapy in thalassaemia to alter significantly the prognosis and life expectancy when he showed that children treated with subcutaneous infusions of DFO achieved negative iron balance.

On the basis of this and other studies showing that urine excretion of ferrioxamine increased with concurrent administration of ascorbic acid (Pippard, *et al* 1978a, Wapnick, *et al* 1969) the recommendation was to start chelation with SC DFO as 12 hr infusions on 5 nights a week as soon as possible in children who were on transfusion programmes along with low dose ascorbic acid (Weatherall and Clegg 1981).

However the benefit of chelation therapy in preventing cardiac failure (Wolfe, *et al* 1985) and the role of intensive chelation in patients with established heart failure (Aldouri, *et al* 1990, Cohen, *et al* 1989) was not established until much later. Since the first studies documenting reversal of cardiac failure in thalassaemia patients DFO has become the gold standard for treatment of acute cardiac decompensation (Davis, *et al* 2004, Davis and Porter 2002).

Despite the very clear benefits of chelation therapy, it is a difficult treatment to use and adherence to therapy has been a major issue for both clinicians and patients

over the last 30 years. Gabutti and Piga (Gabutti and Piga 1996) showed clearly that survival was dependant on the frequency of chelation therapy used. Zurlo and Borgna-Pignatti (Borgna-Pignatti, *et al* 2004, Zurlo, *et al* 1989) showed a reduced frequency of complications due to iron overload in patients who had received chelation from an early age. In addition survival data from the UK registry (Modell, *et al* 2000) showed that despite good access to chelation therapy the outcome was still poor with over 50% of patients dead by the time they reached 35yrs of age. The search for an alternative chelator, which is easier to take, has been the goal for many physicians looking after patients with thalassaemia.

In the search for an effective oral chelator many were investigated but only a few have ever reached clinical studies in humans. I will only discuss the oral chelators that are in clinical use currently and how these have affected thalassaemia management.

1.2.8.2 Deferiprone (1,2,-dimethyl-3-hydroxypyridin-4-one, CP20, L1)

Deferiprone is of the hydroxypyridinone class of chelators and was first identified as a chelator in the late 1980's (Kontoghiorghe and Evans 1985, Kontoghiorghe, *et al* 1987b). It is bidentate and 3 molecules of deferiprone are required to bind one ferric iron. Early studies on humans a mere 2 years after its identification as a chelator of iron showed good urinary iron excretion (Kontoghiorghe, *et al* 1987a). The first study on safety was published in 1992 by Al-Refaie (al-Refaie, *et al* 1992b), who found it to be an effective chelator but with significant side effects including neutropenia and significant arthropathy. Since that time to the present day there have been many publications on its effectiveness and safety (al-Refaie, *et al* 1995, Kontoghiorghe, *et al* 1990, Olivieri, *et al* 1990). However it is also a difficult treatment to adhere to, because of multiple tablets have to be taken three times daily and its significant gastrointestinal side effects. In

addition the very serious side effects of neutropenia/ agranulocytosis require continuous weekly monitoring of blood counts and severe arthropathy which can occasionally be destructive (Ceci, *et al* 2003, Cohen, *et al* 2000, Maggio, *et al* 2002).

Recently there has been much debate in the literature on combination therapy with deferiprone and DFO because of the relatively poor hepatic iron clearance of deferiprone (Caro, *et al* 2002, Mourad, *et al* 2003) , and the possibility that deferiprone may preferentially clear iron from the myocardium (Anderson, *et al* 2002, Hershko, *et al* 2004, Hershko, *et al* 2002, Wu, *et al* 2004).

A full discussion of this debate will not be undertaken because clear clinical evidence for this in the form of randomised clinical trials is still awaited. There is no doubt however that the deferiprone has helped to improve adherence to therapy and improved iron burdens in a significant number of patients taking it who were unable to adhere to DFO therapy.

1.2.8.3 Deferasirox (ExJade, ICL 670)

Deferasirox is an N substituted bis-hydroxyphenyl-triazole and belongs to a new class of synthetic tridentate chelators. 2 molecules of Deferasirox bind to one molecule of ferric iron. It underwent extensive preclinical animal studies and was found to be a potent and selective oral iron chelator. It was well absorbed and tolerated in the phase 1 and 2 studies (Nisbet-Brown, *et al* 2003) and 20 to 25mg/kg doses have been found to be as effective as DFO with regards to hepatic iron clearance and ferritin values (Cappellini, *et al* 2006, Piga, *et al* 2004, Porter, *et al* 2004).

During large phase 2 and 3 studies, side effects which were thought to be associated with the drug were skin rash (10%) transient nausea (20%), diarrhoea (20%) and abdominal pain and vomiting (15% each). Mild rises in the serum creatinine were observed in 30% of patients and these resolved on dose reduction. Serious adverse

events have been rare and no cases have been reported of arthropathy or agranulocytosis. Its impact on survival and adherence is likely to be significant because patients who find chelation with DFO difficult or have serious side effects with deferiprone will now have the option of once daily oral medication. The long-term impact on survival and the incidence of iron-associated complications will become clearer over the next 10 years or so.

1.2.8.4 Optimal use of iron chelators

A challenge with the use of any iron chelator is that although excess iron is potentially toxic, iron is also required for normal physiological purposes. Hence over chelation may result in toxic side effects of iron depletion. From the first reports of falling iron burdens with chelation therapy (Barry 1974) to the present day, the understanding on safe and effective use of DFO in order to minimise drug related side effects such as high tone hearing loss (De Virgiliis, *et al* 1979), spinal column and growth abnormalities (De Virgiliis, *et al* 1988), night blindness and other complications such as cataracts (Bloomfield, *et al* 1978, De Virgiliis, *et al* 1988) has increased significantly. It is now rare to see major toxicity in newly treated thalassaemia patients. The development of the therapeutic index has provided clinicians with a simple method for reducing toxicity and still maintaining adequate iron chelation (Porter, *et al* 1989). The side effects associated with deferiprone require regular neutrophil count monitoring weekly, in addition, liver function tests and serum zinc levels should be monitored regularly and zinc replacement therapy given if necessary. In patients on combination therapy appropriate attention has to be given to the side effects of both drugs. The long term complications from the use of Deferasirox are not yet clear as patients have not been treated for a sufficiently long period. There is however some concern relating to the potential for renal impairment.

1.2.9 Monitoring of Thalassaemia major

Monitoring of young patients to assess for adequate growth and development and x rays to assess bone age, the annual auditory and ophthalmic assessments as well as the use of bone density scans to assess osteopenia/ osteoporosis have allowed clinicians to diagnose problems early and ensure better outcomes for the patients.

Diagnostic tools to look at complications have allowed significant improvements in treatments; for example the use of DEXA scans (dual energy x-ray absorptiometry) for the diagnosis of osteoporosis in thalassaemia patients (Angastiniotis, *et al* 1998) has allowed interventional therapy with bisphosphonates such as Pamidronate (Chatterjee, *et al* 2003b, Morabito, *et al* 2002) to help improve bone density. The monitoring of myocardial iron overload has always been problematic because of the inter observer variability seen with echocardiography (ECHO). The use of MUGA (Multiple gate Acquisition /radionucleotide ventriculography) to provide very accurate information on the left ventricular ejection fraction has allowed appropriate management decisions to be made (Davis, *et al* 2004). The most significant advance in monitoring of myocardial iron load has been made with MRI (Magnetic Resonance imaging) and this will be discussed in chapter 4.

Liver iron burden was always traditionally investigated using liver biopsy but this is now used less frequently because the MRI techniques used can quantify hepatic iron load. Liver biopsy however remains extremely useful, but under utilised, for monitoring fibrosis in patients who are infected with hepatitis C secondary to blood transfusion.

The impact of better transfusion practice and the use of appropriate chelation in the management of TM has transformed the lives of patients in the northern hemisphere

in the 80 years since Cooley's original report. Whereas death was the only outcome for severe TM children in the first decade of life, it is now predicted that a child born today should expect a good quality of life with relatively few iron associate complications (Borgna-Pignatti, *et al* 2004), to have a family, a career and a near normal life expectancy. This however is not the case in many parts of the southern hemisphere where children cannot be given safe blood or be maintained on high transfusion regimes because of lack of resources and the lack of affordable chelators. In these children all the classical features of Cooley's anaemia are still seen as well as the complications of severe iron overload as can be seen in the seventeen year old girl in figure 1.2.9a and 1.2.9b.

Figure 1.2.9.a Under transfused thalassaemia major patient



Legend: A 17 year old girl with TM in Pakistan, receiving chelation with her blood transfusion only. Showing a protuberance of the frontal bones, prominent maxilla and protuberance of her teeth .In addition she is jaundiced. Verbal consent was given for the picture to be taken and used.

Figure1.2.9.b Thalassaemic girl aged 17 years



Legend: Same young girl standing with her mother. Note the stunted growth, lack of sexual development, and slate grey discolouration of the skin in comparison to her mother.

1.3 Thalassaemia Intermedia

TI is a milder form of thalassaemia, which does not require transfusion to maintain life. These children grow normally but will have significant anaemia and will either be transfusion independent, require blood transfusion occasionally or be on regular transfusion regimes at 6 to 12 weekly intervals. Transfused TI patients are described as those requiring less than eight transfusions per annum, if they require more than this then they are re-classified as TM patients.

1.3.1 Historical perspective

In 1925 Rietti published the first description of this milder anaemia in the Italian medical literature, with similar descriptions of children and adults being published by Greppi (1928) and Macheli (1935). It was called the *La Malattia di Rietti-Greppi-Micheli syndrome*. Wintrobe first noted a similar syndrome in 3 Italian families with anaemia and splenomegaly in Baltimore (USA) in 1940. Many workers described similar patients in the American literature and by 1949 it was clear that *La Malattia di Rietti-Greppi-Micheli syndrome* was a form of thalassaemia intermedia.

These patients lived into adulthood but often developed some of the stigmata of Cooley's anaemia. In 1956 the first patient with HbH was identified when a fast moving band was noted on an electrophoretic strip. In 1958 Hb Barts was identified in a 9 month old infant, with a blood film suggesting thalassaemia, at St Bartholomew's hospital. It became clear soon after these discoveries that haemoglobin consisted of α and β

chains and lack of sufficient production of either of these could cause a thalassaemia like syndrome (Weatherall and Clegg 1981).

1.3.2 Molecular and genetic basis of thalassaemia intermedia

The severity of thalassaemia syndromes is determined in general *by the imbalance of alpha to non alpha (beta +gamma) globin chain synthesis*. Factors such as the ability of red cells to destroy unpaired globin chains by proteolysis can contribute to the severity of the thalassaemia syndrome. While it is on many occasions possible to predict a TI phenotype on the basis of the genotype, such as a homozygous mild beta-mutation (such as IVS1–6), there are a number of modifiers of the phenotype that can modulate the genotype such as HPFH or proteolysis of excess unpaired globin.

A TI syndrome may arise from one or more of the following:

- Homozygote (or compound heterozygote) for mild β thalassaemia mutation
- Homozygote for severe β mutation but persistence of HbF due to;
 - Xmn polymorphism
 - HPFH
 - Other factors increasing HbF
- Coinheritance of β thalassaemia (heterozygote) and a thalassaemia-like Hb variant such as;
 - Hb E β thalassaemia
 - Hb Lepore β thalassaemia

These may result in a TM or TI phenotype

- Coinheritance of a thalassaemia mutations with homozygous β thalassaemia
 - Resulting in *decreased* globin imbalance from homozygous α β thalassaemia

- Coinheritance of an extra alpha gene with heterozygous for β thalassaemia
 - (resulting in *increased* globin imbalance compared with β thalassaemia trait)
- Very rarely heterozygote from dominant thalassaemia mutation

1.3.3 Complications associated with thalassaemia intermedia phenotypes

TI is a clinical diagnosis and covers a wide range of homozygous, compound heterozygous and heterozygous genetic defects. The severity of the anaemia is extremely variable and some patients present at a relatively young age and require intermittent transfusion support, other patients have very mild anaemia and are diagnosed in adulthood when a mild anaemia is noted. All patients with TI can develop complications secondary to anaemia. These tend to be more common in the moderate to severe TI patients. These complications are very similar to those described in the TM patients with splenomegaly, venous ulcers, and bone abnormalities on x ray being commonly seen. Venous ulcers are a frequent occurrence in TI patients and have been described extensively in the literature. To date there is no clear consensus on how best to treat these and a range of options have been used including transfusion support, HbF modifiers as well as topical treatments.

Thrombosis is a considerable concern in TI patients and this is more common post splenectomy (Atichartakarn, *et al* 2002) . It is possible this may be associated with the underlying coagulopathy (due fragments of red cell membranes) or the long-standing hypoxia (Aessopos, *et al* 2005, Aessopos, *et al* 2001). Osteopenia and osteoporosis in TI patients are well described (Angastiniotis, *et al* 1998, Dresner Pollack, *et al* 2000, Mahachoklertwattana, *et al* 2003) and the optimal management of this is a yet unclear although there is some data to suggest that TI patients have low bone turnover and respond poorly to bisphosphonates (Chatterjee, *et al* 2003a).

It is well documented that patients with the intermedia syndromes develop iron overload secondary to hypoxia which increased gastrointestinal iron absorption (Celada 1982, Pippard, *et al* 1979, Pippard and Weatherall 1988). Hitherto the possible mechanism for this were unclear but the discovery of iron transporter molecules that are involved in regulation of iron absorption such as DCT1 (Gunshin, *et al* 1997) and the discovery of further molecules involved in iron homeostasis such as ferroportin/Ireg1 (Abboud and Haile 2000, McKie, *et al* 2002) and hepcidin (Park, *et al* 2001) have provided some insight into possible mechanisms.

The management of TI is largely symptomatic and the real advances in management are the ability to transfuse and chelate those patients who develop complications or have symptomatic disease. The most important advance has been the avoidance of splenectomy if possible and to only use this if the spleen is massively enlarged or if there are signs of hypersplenism in order to avoid the thrombosis risk and the use of anticoagulants in those who are at high risk of thrombotic events.

1.3.4 Mechanisms of increased Iron absorption in thalassaemia intermedia.

Blood transfusion is intermittent or absent in TI and the increased iron loading is primarily due to excess iron absorption. The degree of ineffective erythropoiesis, the extent of erythroid expansion and the severity of the anemia all effect the degree of GI iron absorption. The role each factor plays in an individual patient with TI is difficult to predict. Pippard showed in one study that TI patients absorbed 26-73% of food iron (Pippard, *et al* 1979). More recently Pootrakul examined iron absorption in HbE/ β -thalassaemia and found that it varied between 20-75% and correlated with plasma iron turnover, transferrin saturation and liver iron concentration (Pootrakul, *et al* 1988). Iron absorption in thalassaemia intermedia can thus be up to 5-10 times normal, or 0.1 mg/kg/day (Gordeuk, *et al* 1987) .

In TI, the main mechanism of increased iron loading is through increased iron absorption from the diet. Increased iron absorption is also seen in TM but the rate of iron loading from blood transfusion is at least ten times that which results from iron absorption. Other forms of anaemia such as SCA may not be associated with increased iron absorption, possibly because ineffective erythropoiesis is not a prominent feature in this condition, unlike TI. A key goal of this thesis is to contribute to the understanding of how iron loading and distribution can differ between thalassaemia and sickle disorders. This is dealt with in greater detail in subsequent chapters but a description of the factors known to regulate gastrointestinal absorption is given here as an introduction to the subject.

The amount of iron absorbed per day is tightly regulated in humans and over the last few years several key regulators of iron absorption have been identified and their roles defined. The first regulator to be identified was the iron transporter DCT1 (divalent cation transporter) (Gunshin, *et al* 1997) which is known to be expressed on the apical surface of enterocytes and has also been shown to be responsible for uptake of low molecular mass NTBI species in early red cells (Andrews 1999) and is probably responsible for the increased uptake that was seen in cardiac myocytes as described by Parkes (Parkes, *et al* 1993).

Following this discovery attention was focused on the basolateral membrane of enterocytes and ferroportin (Ireg1) was identified (Abboud and Haile 2000, McKie, *et al* 2000). Ferroportin is thought to be responsible for the transport of iron out of the duodenal enterocyte into the plasma compartment where it is bound to transferrin. Then Dcyt b (Duodenal Cytochrome b) was identified by the same group working on ferroportin (McKie, *et al* 2001), it is a ferric reductase that is thought to be responsible for

the reduction of ferric iron to supply ferrous iron to DCT1 on the apical border of the duodenal enterocyte.

The discovery of hepcidin which is a 25 amino acid peptide expressed by hepatocytes and was first isolated from human urine by Park *et al* (Park, *et al* 2001) has been important in providing some insight into regulation of iron homeostasis. The liver was found to be the main site for mRNA expression. Hepcidin and its relationship to anaemia was noted by Nicholas (Nicholas, *et al* 2002a) when transgenic mice over expressing hepcidin died of severe iron deficiency anaemia soon after birth. The relation between anaemia and hepcidin was further defined by Nicholas when mice were subjected to anaemia by repeated phlebotomies to induce iron deficiency or exposed to phenylhydrazine to induce haemolysis. The phlebotomised mice were found to have an 80% decrease in hepcidin mRNA even in the presence of normal liver iron stores and the haemolytic anaemia mouse model was found to have a 3 fold reduction in the presence of an increased liver and serum iron.

Tissue hypoxia secondary to anaemia may therefore play a major role in hepcidin regulation and result in increased iron absorption. More recently work by Adamsky *et al* using a TI mouse that was transfusion independent found reduced liver hepcidin mRNA (Adamsky, *et al* 2004) suggesting that the down regulatory effect of anaemia overrides the up regulatory effect of iron overload. Follow up work from this group showed decreased hepcidin expression in TM mice (Weizer, *et al* 2004). In addition on exposure of human hepatoma HepG2 cells to human thalassaemic sera they found that hepcidin expression was an average of 3 fold lower than that evoked by normal sera. The down regulating effect with thalassaemic sera but not with normal sera suggested that there may be a upstream factor whose levels may increase in the sera in response to ineffective erythropoiesis and that this may override the upregulation of hepcidin in response to iron overload. There has been no study looking at the relationship between

hepcidin, anaemia, iron burden and NTBI in patients with thalassaemic syndromes and SCA. In chapter 5 the relationship between iron overload and hepcidin and its relationship with NTBI in the thalassaemia syndromes and SCA will be discussed.

1.4 The Sickie Syndromes

1.4.1 Genetic and molecular basis of sickle syndromes.

Sickle haemoglobin (HbS) is caused by a single nucleotide substitution in codon 6 (GAG to GUG) of the β chain resulting in the insertion of valine in place of glutamic acid. There are a number of variant haemoglobins (HbC, HbD Punjab, HbO Arab, Hb Lepore etc.) which when co-inherited with HbS result in sickle syndromes. HbS can also be co-inherited with β thalassaemia chain mutation causing (S β Thalassaemia) that results in sickle manifestations of varying severity dependant on the type of β thalassaemia mutation.

The syndromes can be divided into the following subgroups:

- a) Carriers of the sickle haemoglobin are clinically asymptomatic
- b) Homozygous sickle cell anaemia
- c) Compound heterozygote

Homozygous sickle cell anaemia is a very heterogeneous disorder with some patients having severe disease and others mild or moderate disease. HbS can interact with other haemoglobins, resulting in a sickle syndrome. The most common are HbC, HbO Arab, HbD Punjab, Hb Lepore, and β thalassaemia mutations. HbSC tends to have a milder clinical course than HbSS as the haemolysis is less severe but the micro-occlusive events particularly in the eye tend to be more severe. HbSD Punjab is often of

a severe phenotype as is HbS OArab whereas HbSE and HbS Lepore are relatively milder. The severity of HbS β thalassaemia will vary dependant on the β mutation. Those with S β^0 thalassaemia will have as severe a sickle disorder as those with HbSS, whereas those with S β^+ thalassaemia will have a milder clinical course.

1.4.2 Pathophysiology of sickle cell anaemia

The basic defect resulting in sickling episodes is the formation of rigid deformed sickle shaped red cells. The red cells will start to sickle at oxygen saturations below 85% and will nearly all have sickled by 38% oxygen saturation. The red cells become deformed as a consequence of the crystallisation of HbS within red cells (Bertles and Dobler 1969). These cells have increased mechanical fragility and tend to occlude vessels in the microcirculation. The stasis that occurs as a consequence of this, results in further hypoxia and worsening sickling of the red cells and a vicious cycle develops. The tissues distal to the occlusion develops ischemic damage. Vaso-occlusive crises typically occur in bone marrow leading to sporadic bone pain. Other tissues affected acutely by the sickling process include the lungs (chest syndrome) central nervous system (stroke), gastrointestinal and penile tissue.

The sickling process results in shortened red cell survival resulting in a haemolytic anaemia but this does not typically require blood transfusion in the steady state with Hb values typically between 7 and 9 g/dl. . Blood may be rapidly sequestered in the spleen or occasionally liver and the patient will present with worsening anaemia or hypovolemic shock. Hyposplenism resulting from repeated episodes of splenic ischaemia leads to an increased risk of pneumococcal septicaemia. The chronic effects of sickle cell disease may affect many other tissues, such as the kidney (chronic renal

failure), the retina (proliferative retinopathy), bone (avascular necrosis of the hip), the skin (leg ulceration)(see below).

1.4.3 Incidence and frequency of sickle cell anaemia

The initial distribution of the HbS gene was determined by the frequency of occurrence of the mutation and the occurrence of *falciparum malaria*.

Literature published in the 1940's defined it as a predominantly negroid disease:

"A disease essentially peculiar to Negroes, which is characterised by anaemia, rheumatoid manifestations, leg ulcers and acute attacks of pain, and is distinguished morphologically by peculiar sickle-shaped and oat shaped red corpuscles."

(Wintrobe 1946)

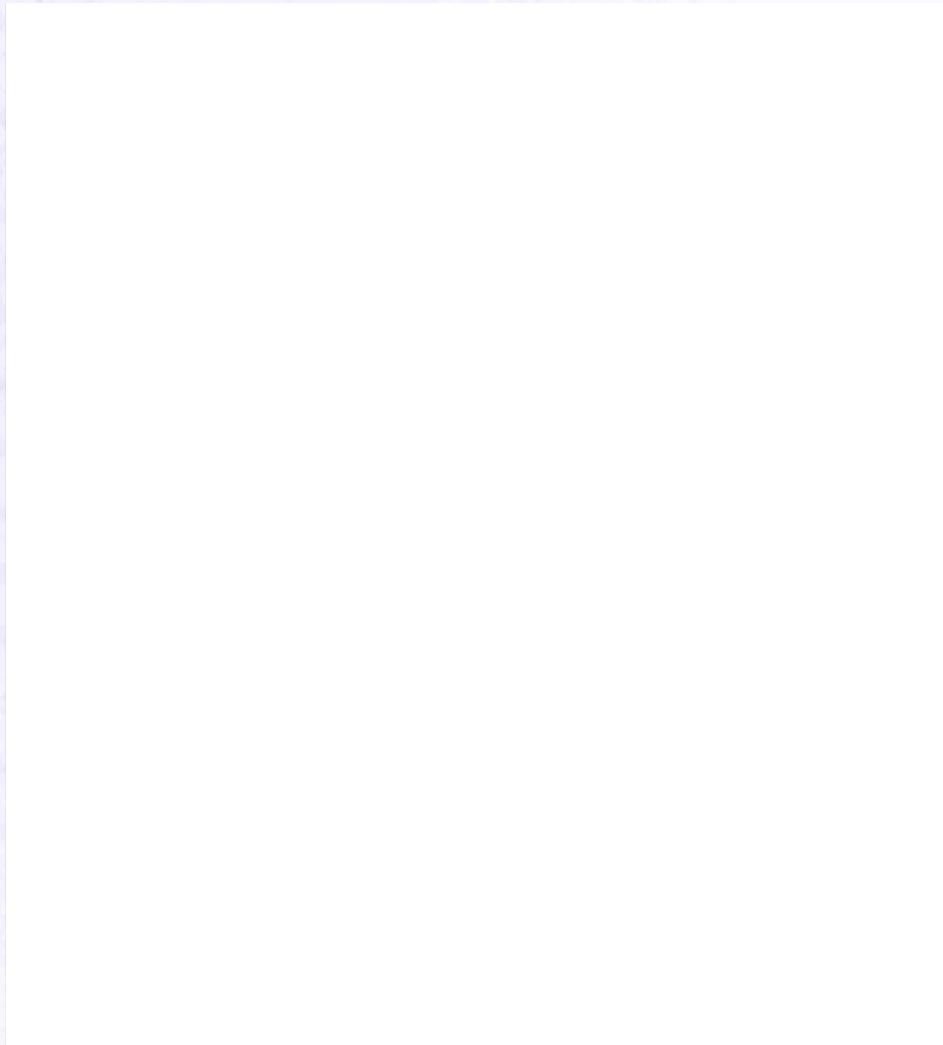
By the 1930's however a number of families had been described in the USA of Greek, Italian and Sicilian ancestry who also had SCA, however this was assumed to be due to "ancestral Negroid blood" . As can be seen from figure 1.4.4a the incidence of distribution of SCA had been quite well documented by the early 1950's.

Figure 1.4.3a Distribution of sickle cell anaemia in the old world

Legend: Figure from Dacie JV, The haemolytic anaemias: part 1 Congenital anaemias. The distribution of Sickle cell anaemia in the Old World. In the early descriptions SCA was thought to be a predominantly Negroid disorder (Dacie 1960)

The distribution of sickle has changed significantly world wide due to migration. In the UK Hickman recently documented the frequency and distribution of SCA. There are approximately 10,000 patients in the UK with SCA and approximately 178 births/annum (Hickman, *et al* 1999).

1.4.3.a Geographical distribution of SCA in England



Legend: reproduced from Hickman (Hickman, et al 1999) the majority of SCA births are in London and the midlands. These are the traditional areas where migrant families settle

14.4. Historical perspective of sickle cell anaemia

Herrick first identified sickle cells in 1910 when he published an article called

“Peculiar Elongated red blood corpuscles in a case of severe anaemia”

The patient was a 20 year old Negro who was a dental student in Chicago and gave no history of rheumatism or other joint trouble. This first case report described the abnormal morphology of the erythrocytes but the description of ‘sickle cell anaemia’ was not used for the disorder until Mason introduced the term in 1922. The carrier state or trait was identified in the parents of patients with SCA by 1923 by Taliaferro and Huck (Dacie 1960). Patients usually presented within the first year of life with anaemia or painful crisis often associated with a febrile course. There would be rheumatic pains and jaundice. These crises were described as thrombotic crises due to agglutination of the erythrocytes (Weatherall and Clegg 1981). Wintrobe gave a classical description of patients with SCA in his textbook describing them as:

“underweight, the trunk is short, the extremities long, the body habitus linear with comparatively narrow hips and shoulders.”.

Wintrobe commented in his textbook Clinical Haematology (Wintrobe 1946) that the majority of children would die in the first decade of life and few would survive to the 3rd decade. Death was usually due to intercurrent infection especially tuberculosis, cardiac failure due to haemorrhage or thrombosis and renal failure. Gallstones (Washburn 1911) were recognised as a problem and death due to shock precipitated by the rapid removal of sickled red cells from the circulation was also documented (Sydenstricker 1924a). By 1960 Dacie in the second edition of his authoritative textbook on haemolytic anaemias, vaso- occlusive crises were described clearly along with osteomyelitis (Hodges and Holt 1951) , sickle stroke (Sydenstricker, *et al* 1923),

pulmonary infarcts (Graham 1924), the risk of death if anaesthetised, and the inability to concentrate urine (McCrory, *et al* 1953). However SCA was still described it as a disease predominantly of childhood (Dacie 1960). By the early 1980's however, our understanding of SCA was very similar to the present day. The prognosis had altered somewhat by this time and this was predominantly felt to be dependant on the socio-economic circumstances of the patient. The management of acute and chronic complications of SCA have changed significantly and the use of blood transfusions has become more widespread.

1.4.5. Complications of SCA that may require blood transfusion.

A detailed description of all the complications of sickle cell disease and their management is beyond the scope of this chapter, the aim of which is to focus on conditions where repeated blood transfusion may result in iron overload. Below the common complications of SCA for which blood transfusion may be required are discussed.

1.4.5.1. Neurological complications

Stroke was recognised in early reports (Sydenstricker, *et al* 1923) and a review by Hughes in 1940 (Hughes, *et al* 1940) defined the clinical syndrome as occurring mainly in children below the age of 10 years, the frequency of recurrence and the resultant hemiplegia and convulsions. Further reviews have improved our understanding of sickle stroke syndromes and the pathology behind them. Management of the acute stroke syndrome with blood transfusion improved clinical outcomes (Sarnaik, *et al* 1979) but it was not until transcranial Doppler (TCD) was used to monitor cerebral flow in

children that significant improvement has occurred in identifying children at high risk (Adams, *et al* 1998b). This was a benchmark study (known as the STOP1 study) and had considerable impact on patient management. The very important conclusion from this study was that patients with TCD greater than 200 cm/s should receive blood transfusion to reduce the risk of stroke. The cooperative study on sickle cell disease also identified other five other risk factors important in cerebral infarction risk assessment such as recent and frequent chest syndromes, low haemoglobin, recent transient ischemic attack and high blood pressure. In the very young children, dactylitis, high WBC count and significant anaemia were important predictors of severe outcomes (Ohene-Frempong, *et al* 1998). Children with raised TCDs should be started on transfusion programmes aiming for a HbS% of 30% or less as primary prevention. However around 60% of children with abnormal TCDs do not develop stroke even in the absence of transfusion (Abboud and Atweh 2006).

More recently silent infarcts have been noted on 17% of sickle children (Kinney, *et al* 1999). Management of this is unclear but is being evaluated in a clinical trial (Kirkham, *et al* 2006) looking at the use of prophylactic blood transfusion.

Role of blood transfusion:

There are significant uncertainties as to when it is safe to stop blood transfusion in children who have has a stroke syndrome and are receiving blood as secondary prevention with conflicting reports in the literature (Rana, *et al* 1997, Wang, *et al* 1991). In children receiving blood transfusions for primary prevention the STOP1 study showed that if transfusions were stopped once TCDs had reversed into the normal range, these reverted to abnormal values and there was a high risk of stroke (Adams and Brambilla 2005). Not all children with raised TCD's however go onto have a stroke syndrome and these children are facing long term transfusion programmes with the need for chelation

therapy for iron overload. There is a clear need for a more precise risk assessment for sickle stroke syndromes.

1.4.5.2. Acute chest syndromes (ACS)

Chest syndromes can be precipitated by a number of conditions such as pulmonary emboli or chest infections and has been the subject of two large studies the CSSCD (Vichinsky, *et al* 1997) and the MACSS (Vichinsky, *et al* 2000). The syndrome is often not recognised early enough and hence many large centres have devised protocols for the management of this. Evidence of acute respiratory distress with tachycardia, hypoxia and a chest X ray showing pulmonary infiltrates should act as a precipitant for exchange transfusion (Vichinsky, *et al* 2000) and respiratory support either in intensive care or on the ward. Most patients with ACS will receive exchange transfusions or less frequently top up transfusions to help improve the Hb and oxygen carrying capacity. Many case reports have been described in the literature but in the largest series by Wayne (Wayne, *et al* 1993) 32 of 35 patients had rapid improvement in oxygenation and good clinical outcomes. The positive impact on outcomes of blood transfusion on ACS has resulted in many clinicians considering it for use in the prevention of recurrences in patients with severe or repeated episodes. This is primarily due to concerns that recurrent ACS may contribute to chronic pulmonary failure, pulmonary hypertension and cor pulmonale (Collins and Orringer 1982, Powars, *et al* 1988), however the effect of blood transfusion on prevention of these late pulmonary outcomes is unknown because this has not been studied formally. Hydroxyurea has been found to be beneficial in prevention of recurrent ACS (Charache, *et al* 1995).

1.4.5.3. Priapism

Priapism was originally noted as a complication of SCA by Diggs and Ching (Diggs and Ching 1934). Reviews published in the 1960s identified that priapism tended to affect the younger patients and a diverse range of treatments were ineffective (Sousa, *et al* 1962). More recent data (Emond, *et al* 1980) suggested that priapism is more frequent than previously thought and occurred in 40-50% of patients above the age of 20 years, and there are two distinct types (stuttering and acute).

Treatment for priapism continues to be difficult and there is limited and contradictory data on transfusions and possible complications such as ASPEN syndrome (Siegel, *et al* 1993) (Baruchel, *et al* 1993, Miller, *et al* 1995, Rackoff, *et al* 1992). Intracavernosal injections with alpha agonists such as etilefrine or phenylephrine (Muruve and Hosking 1996, Virag, *et al* 1996) have helped improve outcomes to some degree. However a significant number of patients will proceed to surgery to create shunts and eventually to insertions of penile prostheses to help treat priapism (Maan, *et al* 2003, Sadeghi-Nejad and Seftel 2002).

1.4.5.4. Aplastic crisis

This was first recognised as a complication in 1950 (Singer, *et al* 1950). Classically the syndrome presents with symptoms of a recent upper respiratory tract infection followed by a rapid fall in haemoglobin and a reticulocytopenia. Spontaneous recovery occurs within 5 to 10 days and provided the anaemia is not too severe managed conservatively. Occasionally transfusion support is needed in which case the slow administration of one or two transfusions aimed at raising the hemoglobin by 2-3

g/dL over four to eight hours, should be sufficient to improve the clinical condition of the patient until spontaneous recovery occurs.

The association with parvovirus was first recognised by Pattison (Pattison, et al 1981).

1.4.5.5. Splenic sequestration

This was first recognised as a complication in SCA by Tomlinson (Tomlinson 1945) and was found to be the most common cause of death in early life (Rogers, et al 1978). The disorder is of varying severity from mild splenic enlargement and a slight fall in Hb to severe hypovolemic shock (Topley, et al 1981). The early recognition of splenic sequestration crises in children has helped to reduce the mortality from this complication (Emond, et al 1985). It has now become part of routine management to teach parents to recognise splenomegaly and the signs and symptoms of sequestration crisis. This has resulted in a much improved survival in patients (Lee, et al 1995). Management remains transfusion support primarily in the acute setting. Chronic blood transfusion support may reduce the risk of recurrent sequestration crises, reduced splenomegaly and hypersplenism but it does not ultimately prevent the need for splenectomy. It may therefore have a role in delaying splenectomy in children until they are over 5 years of age.

1.4.5.6. Surgery

SCA patients have a high risk of post-operative complications and about a 1% mortality rate whilst undergoing surgical procedures such as cholecystectomy (Koshy, et al 1995), the most frequent complication being ACS. Koshy et al showed that sickle complications were reduced by about sixty percent in those patients who had peri-operative transfusions in low risk procedures. Despite this however the peri-operative use of blood transfusions to prevent complications has been controversial. Some

investigators have used transfusions to simply correct the anaemia and others have attempted to reduce the HbS%, whilst others have preferred to avoid transfusion and pay careful attention to hydration and oxygenation for successful outcomes (Serjeant 1997). A randomized transfusion study in patients undergoing cholecystectomy showed an overall 1% mortality with a 19% complication rate and 5% mortality in those receiving 'no transfusion'. There was no difference in the complication rates between those randomized to aggressive or simple transfusion regimens (Haberkern, *et al* 1997) . Vichinsky *et al* showed that there was no difference in outcome post operatively in patients who were randomised to have exchange transfusions and a reduction on the HbS to below 30% and those who had a top up transfusion to raise the Hb to 10g/dl. (Vichinsky, *et al* 1995).

In those patients who are deemed to be at high risk of complications post-operatively such as those with chronic lung disease or undergoing prolonged surgical procedures exchange transfusions may be more beneficial but there is no data from prospective clinical trials to confirm this currently.

1.4.5.7. Pregnancy

The use of blood transfusion during pregnancy to affect clinical outcomes is controversial with much conflicting evidence in the literature on prophylactic transfusions (Charache, *et al* 1980, Morrison and Wiser 1976, Tuck, *et al* 1987). In the study by Tuck *et al* no beneficial effect was seen in either maternal or foetal outcome. It is generally felt however that pregnant women who develop complications such as pre-eclampsia, septicaemia, acute chest syndrome, exacerbation of anaemia, stroke should receive transfusion (Koshy and Burd 1991) and most clinicians will also tend towards using blood transfusions prophylactically in women with a bad sickle history, bad obstetric history or where the obstetric risk is judged to be high e.g. multiple pregnancy or high risk medical conditions such as diabetes.

1.4.5.7 Chronic conditions that may benefit from transfusion support

Conditions that may benefit from supportive transfusion regimes include pulmonary hypertension, chronic renal failure and venous ulceration. Pulmonary hypertension (PHT) was first reviewed as a complication by Collins and Orringer (Collins and Orringer 1982) where three previous cases were described along with three new cases and this provided the initial guidance on diagnosis and treatment. The prevalence is quoted as being between 30 and 56% (Gladwin, *et al* 2004, Simmons, *et al* 1988). The exact pathophysiology of PHT is not clear but is likely to be multifactorial and includes recurrent chest syndromes, pulmonary emboli, haemolysis and chronic hypoxia (Siddiqui and Ahmed 2003). ECHO is a useful for screening and monitoring and a TR jet velocity of greater than 2.5m/s is suggestive of PHT, however, the frequency of ECHO monitoring is not clear. The gold standard confirmatory test is cardiac catheterisation with measurement of right heart pressures. Treatment remains unclear with a variety of options being used in most patients including anticoagulation (Gaine 2000) , blood transfusion, calcium channel blockers (Rich, *et al* 1992) continuous nocturnal oxygen if responsive on catheter studies , inhaled nitric oxide (Hasuda, *et al* 2000) , hydroxyurea (Castro 1996), vasodilators, prostaglandins and endothelin receptor antagonists (Gladwin and Kato 2005)

Sickle nephropathy has been recognised for a long time from early reports on hyposthenuria (Herrick 1910, Sydenstricker 1924b), tubular dysfunction is well documented and has been recently reviewed ((Ataga and Orringer 2000)). Acute renal failure is seen in patients who are severely septic, are very febrile or have rhabdomyolysis, and is often part of an acute multiorgan failure syndrome. More recently concern has been focused on prevention of end stage renal disease and this has

resulted in regular monitoring of proteinuria and blood pressure in patients at least annually. In those patients with significant proteinuria the use of ACE inhibitors (Powars, *et al* 1991) has been of some benefit in reducing the severity of proteinuria. It is important to control hypertension and avoid NSAIDs (Kontessis, *et al* 1992, Nissenson and Port 1989). However despite these measures patients will often proceed to renal dialysis or renal transplantation. This cohort of patients will often need long term top up blood transfusion regimes to support their anaemia.

The management of avascular necrosis and arthropathy (Almeida and Roberts 2005) and venous ulcers remains difficult (Chung, *et al* 1996) and are the cause for a great deal of morbidity. The healing of sickle venous ulcers may improve with blood transfusions but not all ulcers respond. Iron overload may develop insidiously in this group as transfusions will be for short periods of time and will occur intermittently throughout a patient's life.

1.4.6. Transfusion regimes used in SCA

The administration of one or two units of blood as a top up transfusion on a three or four weekly schedule will reduce the Hb S level to around 30-50%. Exchange transfusion performed either manually or automated on a cell separator will result in less blood being needed to reduce the HbS% by combining the administration of donor red cells with the removal of the patient's red cells (Cohen, *et al* 1992). Exchange transfusion has been found to be extremely useful in reducing the severity of iron overload in patients with SCA on long-term transfusion programs for prevention of stroke or other complications. New iron accumulation is stopped or profoundly reduced. A single Exchange transfusion is also useful in reducing the Hb S% in patients with acute complications or pre-operatively.

1.4.7. Iron metabolism and iron overload in sickle cell anaemia

Although patients with SCA are anaemic, they rarely develop iron overload secondary to increased gastrointestinal iron absorption even when the degree of anaemia is significant (O'Brien 1978). This is probably because the anaemia is primarily a haemolytic anaemia, although some degree of ineffective erythropoiesis is present (Finch, *et al* 1982, Wu, *et al* 2005).

Repeated simple blood transfusions will however result in iron overload, but exchange transfusions does not result in significant iron overload developing particularly if the transfusional iron input is matched with that removed by venesection during the exchange (Porter and Huehns 1987) or by automated red cell exchanges (Cohen, *et al* 1992). Cohen *et al* (Cohen, *et al* 1992) showed in that increasing the pre transfusion HbS% to 50% reduced blood requirements in patients by a quarter in those SCA patients with no recurrence of stroke and no progressive neurological deterioration during at least four years of conventional transfusion.

Even where patients have been heavily transfused, cardiac and endocrine manifestations are infrequently seen. It has been proposed that iron distribution may be different between patients with SCA and thalassaemia and that this may be due in part to the inflammatory response seen in sickle cell disease (Finch, *et al* 1982, Harmatz, *et al* 2002) . There are significant changes in the cytokine profiles in patients with SCA both during the acute vaso-occlusive episode and in the steady state when the patient is clinically well. It was shown by Taylor *et al* (Taylor, *et al* 1997) that type II cytokines such as IL4,6, and 10 were raised in SCA during the steady state. Other workers have also

found raised levels of IL6 but not the other type II cytokines (Bourantas, *et al* 1998, Croizat 1994). More recently Pathare *et al* (Pathare, *et al* 2004) has shown increased IL1 β in the steady state. In addition they looked at the cytokines during acute vaso-occlusion and found increased levels of IL6 and IL8.

It is known that IL6 is a potent inducer of hepcidin (Nemeth, *et al* 2004). Hepcidin down regulates iron absorption from the duodenal enterocytes and also stimulates increased iron uptake by the macrophages (Nicolas, *et al* 2001). It is therefore possible that sequestering of iron in the reticuloendothelial cells such as the macrophages during steady state in SCA results in less exposure to toxic forms of iron such as NTBI. This would therefore result in fewer complications from the hydroxyl radical generation via the Fenton and Haber Weiss reaction (Nicolas, *et al* 2001).

This interesting observation will be discussed in more detail in chapter 5 where the relationship between prohepcidin and anaemia / iron overload in thalassaemia syndromes and SCA will be discussed. In addition the distribution of iron overload particularly in those patients who have transfusional iron load appears to be different. Myocardial iron overload is rarely seen in patients with sickle syndromes, and why this may be so will be discussed in chapter 4.

1.6. Conclusions

The advances in our knowledge and the better understanding of the thalassaemia and sickle syndromes, the most common of all genetic disorders, has allowed better clinical management of patients to the extent that it is now expected that the vast majority of optimally treated patients will have near normal life expectancy.

Over the last 100 years advances in transfusion practice such as the use of ABO and Rh matched blood, the use of packed cells rather than whole blood, virological

screening to reduced the risk of transfusion transmitted infections, leucodepletion and /or the use of washed cells for transfusion in those with persistent reactions has resulted in greatly improved blood safety for the all patients who require transfusion support. The advances in chelation therapy have improved the prognosis and reduced morbidity from iron overload in the majority of patients.

The relationship between NTBI and iron mediated damage has only been assessed in small studies and the time at which NTBI appears in children who have not been transfused or have initiated transfusions is not clear. NTBI is known to be responsible for cell damage via lipid and protein peroxidation but the role these might play in well transfused and chelated patient is unknown. The distribution of iron overload is thought to be different in SCA compared to thalassaemia and these patients rarely developed iron overload secondary to increased gastrointestinal absorption. All of these questions will be reviewed in this thesis to assess what relationship if any exists between NTBI and iron mediated damage in the thalassaemia syndromes and SCA.

Chapter Two

Methods

2.1 Patients

All adult patients gave verbal consent for their samples to be taken and stored for the research studies. The consent for the children was written consent from parents or guardians. The liver biopsy samples were historical samples from thalassaemia patients and where possible verbal consent was taken for the biopsy samples to be used for the measurement of hepcidin.

2.1.1 Study sites

The children were from a number of centres. There were 28 children from a National Thalassaemia Centre in a town called Kurunegala in Sri Lanka where ethical approval had been granted. These patients were managed by the local doctors and advice on management provided by Dr Nancy Olivieri and Professor Sir David Weatherall. There were 21 children from the Hospital for Sick Children (HSC) in Toronto, Canada who were under regular review and 2 children both born at UCLH in the study

from the main centre. The adult patients in the studies were all patients who were regularly treated at UCLH, London.

2.1.2 Patient cohorts

All the children had thalassaemia major or intermedia and numbers were as in section 2.1. The adults comprised 26 patients with sickle cell anaemia, 76 TM and 23 with TI.

2.1.3 Ethics

The local ethics committee at UCLH gave ethical approval for all the studies. For the studies involving children the local ethics committees at HSC, Toronto, Canada and the Sri Lanka centre gave the approval for the study enrolment

2.2 Sample Handling

All blood collections were undertaken when the patient had received no desferrioxamine for 48 hrs and were clinically well.

2.2.1 Sample Processing

7 mls of blood was collected in haemogard vacutainer tubes (Becton Dickinson). All samples were left standing at room temperature for 30 minutes (it was necessary to leave them for slightly longer in patients on anticoagulants but not beyond 1h).

After 30 minutes, samples were centrifuged at 3000 rpm for 10 minutes at 4°C in pre-cooled centrifuge. 0.4ml aliquots of serum were frozen in freezing tubes and labelled with the date and time of sampling.

2.2.2 Sample Storage

All blood samples were stored at -80°C freezer until ready for analysis. The maximal storage of samples prior to analyses was within a year.

2.3 Non Transferrin Bound Iron (NTBI)

2.3.1 Principle

Once transferrin becomes saturated in iron overloaded patients, free iron is thought to form oligomeric and monomeric complexes of iron with ligands such as citrate, phosphate and albumin resulting in NTBI (Hider 2002, Jacobs, *et al* 2005). NTBI was measured using a method based on iron mobilisation on to NTA followed by separation of mobilised iron using ultrafiltration. The NTBI was then detected by HPLC using the method described by Singh *et al* (Singh, *et al* 1990).

Samples

It is not known if NTBI alters if samples are kept at room temperature, or in a normal fridge and for this reason we did not use samples that had been defrosted in transit to the UK. Bacterial contamination is a concern and is another reason why samples were kept frozen.

Normal range: for the normal control this value should appear negative. Reference interval (95%) for n=29 normal subjects was -0.61 ± 0.90

For iron overloaded serum this would have a positive value, reference interval (95%) for n=80 iron overloaded subjects was 4.52 ± 3.69

2.3.2 Method

0.18 mL of serum was incubated with 0.02 mL of 800 mM NTA at pH 7.0 for 30 min at room temperature to form the Fe-NTA complex. Proteins and other higher molecular mass materials were removed from the serum by filtration through Whatman Microfree Ultracentrifugation devices (molecular mass cut-off 30 K) at 10,500 rpm, 30 min, 4°C. 25 µL of the filtrate was then injected onto a C18 reversed phase HPLC column (Varian: glass- no metallic components) equilibrated to 5 mM MOPS containing 19% acetonitrile and 3 mM 3-hydroxy-1-propyl-2-methylpyridin-4-one (CP22) at pH 7.0. The Fe-NTA delivers the iron to the CP22 to give a red coloured complex, which can be quantitated by comparison with Fe-NTA standards.

After every 5 samples, the 2 and 8 µM standards were run to check for diminution in peak height and all the standards were run again at the end of the experiment. Standards and samples were integrated, extracting chromatograms at 430 nm. The standard peak height versus concentration was plotted and concentration of sample was calculated from its peak height using the graph.

2.4 Transferrin Saturation

2.4.1 Principle

This was determined using urea polyacrylamide gel electrophoresis, which resolves all the three forms of iron-loaded transferrin from apotransferrin. The percentage saturation was calculated by scanning the bands (Evans and Williams 1978). The presence of NTBI, ferritin iron, plasma pigments and high levels of bilirubin can results in inaccurate measurements at high transferrin saturations using the routine clinical method (al-Refaie, *et al* 1992a).

2.4.2 Method

25 μ L of each serum sample was treated with a solution of rivanol (6.9-diamino-2-ethoxyacridine lactate: 94 mg in 25 mL of aqueous 0.1 M tris; 0.01 M borate; 1.6 mM EDTA buffer at pH 8.4 containing 8 % glycerol) to precipitate all serum proteins except the γ -globulins. The protein precipitate was removed by centrifugation at 13,000 rpm for 5 min after which 55 μ L of the supernatant was loaded onto a 6 % acrylamide gel formed in 6 M urea and electrophoresed at 110 volts for 20 hours. The colour intensity for the four resolved transferrin bands was determined by scanning and the percentage saturation was calculated using the formula:

$$\% \text{ Saturation} = \frac{(2 \cdot I_{\text{diferric}} + I_{\text{c-mono}} + I_{\text{N-mono}}) \cdot 100}{2 \cdot \Sigma (I_{\text{diferric}} + I_{\text{c-mono}} + I_{\text{N-mono}} + I_{\text{apo}})}$$

2.5 Soluble Transferrin Receptor (sTfR)

2.5.1 Principle

Soluble transferrin receptors can be measured in serum by an immunoturbidometric method. The sTfR antibodies are coated onto Latex particles and in the presence of sTfR, agglutination occurs in a dose dependant manner. The increased turbidity is detected at a wavelength of 600nm on a Cobas MIRA. The results are calculated from a reference curve.

2.5.2 Reagents

BioStat IdeA sTfR-IT- Soluble transferrin Receptor (sTfR) Assay Cat. No. 67968

The IdeA sTfR-IT Kit has been standardised against a monoclonal immunoassay (EIA) method.

Additional Reagents:

Cat. No. 67976 IdeA sTfR-IT- Control Low

Cat. No. 67975 IdeA sTfR-IT- Control High

Norma range with assay: 0.3-8.5 mg/l

2.5.3 Method

Sample analysis was carried out in duplicate using a Cobas Mira. IdeA sTfR-IT- Reagent was reconstituted with 4ml of deionised water, mixed gently and left to stand at room temperature for at least 60 minutes. This was mixed once again prior to use and

was used as start R1. IdeA sTfR-IT-Buffer was used as “reagent”. The calibrators that came with the kit were IdeA sTfR-IT-calibrators were used to calibrate and PBS was used as standard point 1 (0 mg/l). IdeA sTfR-IT-Controls were used. The results were analysed on a Cobas MIRA (Roche Diagnostics) at a wavelength of 600nm and the results calculated from a reference graph.

2.6 Serum Protein Carbonyls

Protein carbonyls are derived as a result of hydroxyl radical induced oxidation of the amino acid side groups of proteins. This results in the formation of intra-protein aldehydes and these can then initiate cross linking by reacting with ϵ -amino groups. These are discussed in more detail in chapter 3.

2.6.1 Principle

Protein carbonyls were measured by a modification (Carmine, *et al* 1995) of the original method described by Reznick (Reznick and Packer 1994). Carbonyls react with dinitrophenylhydrazine (DNP) dissolved in hydrochloric acid and the protein-carbonyl - DNP complex is then precipitated with trichloroacetic acid (TCA). The precipitate is extensively washed to remove excess DNP and then dissolved with guanidine hydrochloride and its absorbance measured at 380 nm.

Normal range in our lab in healthy adult control subjects 0.72 ± 0.27 nmol/mg.

2.6.2 Method

The samples were diluted in PBS and to this was added 10mM DNP dissolved in 2M HCL. The complex that was formed was precipitated with TCA and washed 3 times with ethanol/ethyl acetate mixture (1:1). The pellets were broken up between each washing using a spatula and the new pellet reformed by centrifugation. The final pellet was dissolved in 6M guanidine HCL and the absorbance at 380 nm was measured. Blank controls consisting of the sample and 2M HCL only were taken through the same procedure. The absorbance for these at 380 nm was used for controls, and absorbance at 280 nm used to measure the protein concentration of the sample. The carbonyls values were expressed as nmol/mg of protein.

2.7 Malondialdehyde (MDA)

2.7.1 Principle

MDA analyses were undertaken using the HPLC method as described by Halliwell and Chirico (Halliwell and Chirico 1993). Amplification of peroxidation during the heating process was prevented by adding the chain breaking antioxidant butylated hydroxytoluene (BHT) to the sample before the remaining reagents were added. HPLC was used to separate the authentic $(TBA)_2$ -MDA adduct from other chromogens absorbing at 532 nm.

Normal range in our lab in healthy adult control subjects was 0.27 ± 0.22 μ M (range 0.03-0.68 μ M)

2.7.2 Method

The HPLC column was equilibrated for 2 –3 hours prior to start of assay with the mobile phase consisting of 65% 50mM KH_2PO_4 and 35% methanol. 0.2% BHT was added to serum and standards and then divided into equal aliquots. These were incubated with 0.44M phosphoric acid for 10 minutes following which 0.6% TBA was added. The mixture was kept at 90 °C for 30minutes. Once cooled the samples were analysed on HPLC using a techsphere 80 ODS2 column and guard. The calibration curve from the peak height of standards was used to determine the MDA concentration of samples.

2.8 Serum Erythropoietin

2.8.1 Principle

The erythropoietin is measured using a standard ELISA. The wells are pre-coated with a mouse monoclonal antibody against EPO. Once the unbound serum is removed a second incubation with Anti EPO rabbit polyclonal antibody bound to horseradish peroxidase is performed. This incubation results in the antibody-enzyme conjugate binding to the EPO. Once the complex is formed and unfixed conjugate removed, chromogen is added to the wells and is oxidised by the horseradish peroxidase. This reaction is stopped by the addition of acid. The colour change is directly proportional to the amount of EPO in the sample.

2.8.2 Reagents

Quantikine®IVD® erythropoietin ELISA; R&D Systems Inc. Catalog Number DEP00

All reagents were bought to room temperature before use.

Wash Buffer was warmed to room temperature and gently mixed until crystals had dissolved. 100 ml of wash buffer Concentrate was diluted in deionised water to prepare 2500 ml of wash buffer.

Substrate Solution 1 and 2 were mixed together in equal volumes within 15 minutes of use.

The normal range using serum for this assay is 3.3-16.6 mIU/ml

2.8.3 Method

All reagents and specimens were bought to room temperature before use and tests were carried out in duplicate. 1: 10 dilutions were used for samples.

Samples were added to each well following which the plate was incubated at room temperature for 1 hour on an orbital microplate shaker. The wells were aspirated dry and Epo Conjugate was added. A second hour-long incubation was carried out on an orbital plate shaker. Each well was washed 4 times and blotted dry. Following this Substrate solution was added *within 15 minutes of preparation* and a short incubation for 20 minutes performed. At the end of this Stop solution was added and the optical density determined using a microplate reader set at 450nm.

2.8.4 Calculation of results

Duplicate readings were averaged and a standard curve of optical density on the y-axis against the concentration on the x-axis was plotted and the value calculated from the graph.

2.9 C Reactive Protein (CRP)

2.9.1 Principle

The CRP is measured using an ELISA technique. The wells are coated with rabbit antibodies to human CRP. Once the antigen-antibody complex forms following incubation with sera, the unbound serum is removed and a second incubation is performed using a conjugate of peroxidase with anti human CRP antibody. After unbound conjugate is removed the strips are incubated with tetramethybenzidine and hydrogen peroxide. A blue colour develops which is proportional to the amount of bound complex. The reaction is stopped with sulphuric acid and absorbance read at 450nm.

2.9.2 Reagents

CRP ELISA, DRG Instruments GmbH, Germany (EIA-1952).

All samples and reagents were brought to room temperature prior to testing.

The normal range using serum for this assay is 1-5 ug/ml

2.9.3 Method

Samples were diluted 1:1000 for this assay as instructed in manufacture's protocol. The calibrators were diluted to 1:100. Tests were performed in duplicate. 100ul of the diluted calibrators and samples were loaded into each of a pair of wells. The plate was covered and incubated for 30 minutes at room temperature. The strips were then washed three times with wash solution, leaving the solution in the well for 2 to 3 minutes for the first wash. Following this, 100ul of conjugate was added to each well and then incubated for another 30 minutes at room temperature. Washing was repeated as before and then 100ul each of solution A and B was added to the wells. Following a 10-minute incubation 50ul of 2N sulphuric acid was added and absorbance read at 450nm within 30min of adding the acid.

2.9.4 Calculation of results

Duplicate readings were averaged and a standard curve of optical density on the y-axis against the concentration on the x-axis was plotted. The value for the CRP was read by simple interpolation from the graph.

2.10 Serum Pro-hepcidin

2.10.1 Principle

Pro-hepcidin levels were measured using an ELISA technique. The wells were coated with rabbit antihepcidin antibody. Samples and controls were incubated and the antigen antibody complexes were detected using a streptavidin-peroxidase with the

substrate tetramethylbenzidine. The colour reaction was stopped with the addition of acid and the colour change read at 450 nm.

2.10.2 Reagents

DRG Hepcidin Pro-hormone ELISA EIA-40155

The assay is temperature sensitive! The manufacturer advises that the assay is performed at room temperature (18 – 25 °C) only. In our experience the assay is best performed at around 20-22 °C.

Lyophilized contents of the standard /control vials were reconstituted with 1.0 ml bidist. Water.

Wash Solution: was prepared by adding deionized water to a final volume of 1200 ml. All reagents and samples were brought to room temperature before beginning the test. The assay was performed with a 1:1 dilution throughout.

Normal range: 56.1 -156 ng/ml

2.10.3 Method

Pro-hepcidin Standards and Controls were dispensed into appropriate wells and duplicate samples were placed into the remaining wells. Biotin Conjugate was added and the plate mixed thoroughly followed by Incubation for 60 minutes at room temperature. The wells were washed 3 times with diluted Wash Solution and Streptavidin HRP Complex was then added to the wells and Incubated for 30 minutes. This was followed by repeat washings. Substrate Solution was added to each well and incubated for 30 minutes and then Stop Solution was added and the absorbance of each well determined at 450±10 nm.

2.10.4 Calculation of results

The Pro-hepcidin value of each sample was obtained as follows :

Using semi log graph paper, a standard curve was constructed by plotting the average absorbance (Y) of each Reference Standard against its corresponding concentration (X) in ng/ml.

The average absorbance of each sample was used to determine the corresponding Pro-hepcidin value by simple interpolation from this standard curve, multiplying by the initial sample dilution, if necessary.

Limitations of this assay:

There are several pitfalls and limitations with this assay. We initially ran the assay without any dilution and values were found to be on the non linear portion of the curve making the results meaningless. The assay was then repeated using a 1:1 dilution and the absorbance results were then on the linear part of the curve. The other limitation is the temperature sensitivity. We performed this assay in two batches on two separate days and were careful to ensure that the temperature in the lab was around 20-22 °C. The results however between the controls which were the same on both days were all within the normal range although there is considerable variability within the normal range.

2.11 RNA extractions

2.11.1 Principle

Paraffin sections are deparaffinised using xylene and ethanol washes followed by protease digestion to release the RNA.

2.11.2 Reagents

Ambion kit (RNA isolation kit cat. No. 1902)

Proteinase K buffer, proteinase K reagent and linear acrylamide were supplied in the kit.

All other reagents tubes and pipettes used were RNA free products.

2.11.3 Method

10 X 5 μ m sections (more for biopsies, fewer for large pieces) were cut and placed into a 1.5ml tube. 1ml of xylene was added to these and they were mixed until all the wax dissolved. The mixture was then spun at 7000 rpm for 5min following which the xylene was aspirated with a pipette. 1ml 100% ethanol was added to the residue and thoroughly mixed. This was then spun at 7000 rpm for 5min and the ethanol removed with a pipette and this step repeated again. Once the final ethanol was removed the residue was allowed air dry at room temperature or at 65°C. To the dried residue was added 100 μ l proteinase K buffer + 5 μ l proteinase K, and incubated at 45°C for 2 hours. The tubes were spun briefly, 600 μ l of RNA extraction buffer was added and the tube vortexed 5 X 5s. After a 5 minute incubation this was then spun at 7000 rpm for 3 minutes following which 700 μ l phenol/chloroform was added to the mixture and vortexed 5 X 5s. A final spin was performed after 5 minutes incubation. The aqueous phase (top layer) was transferred to a fresh tube, 1 μ l of linear acrylamide added, mixed briefly followed by 1 volume (approx. 600 μ l) of isopropanol. This mixture was left at -20°C overnight following which the sample was spun at 13000 rpm for 15min at 4°C and the isopropanol carefully removed. 700 μ l of 70% ethanol (cooled to -20°C) was added and

spun again. The ethanol was removed and the tube air dried for about 20min before adding 20µl RNA storage solution and storing in a -80 freezer.

PCR was undertaken for hepcidin and this is described in more detail in chapter 5 section

2.12 Cardio magnetic resonance imaging (CMR)

2.12.1 Principle

Iron is paramagnetic and by virtue of this can be quantified by techniques such as MRI. MRI uses either T1 (longitudinal) or T2 (transverse) weighted relaxation times and the presence of iron results in a reduction of the signal intensity. T2 relaxation times therefore become shorter in the presence of iron. The MRI methodology adopted is that used by Anderson et al (Anderson, *et al* 2001) and is described in more detail in chapter 4.

2.12.2 Method

For the measurement of myocardial T2*, a single short axis midventricular slice was acquired using a gradient echo sequence on a Siemens Sonata 1.5T MRI scanner at nine separate Echo times (5.6 –18.0ms.). A gating delay time of 0 ms after the R wave was chosen so as to obtain images in a consistent position in the cardiac cycle and a full thickness region of the intraventricular septum was analysed. For image signal analysis, in house software (CMRtools ©Imperial College) was used.

For the measurement of liver T2* a single transaxial 10mm slice through the center of the liver was acquired using a gradient echo sequence at eight different echo times (2.3 - 20.0ms).

2.13 Statistical analysis

All data analyses were carried out using Microsoft Excel 2003. The mean, median, Standard deviation and Standard Error of the mean were calculated. All results were expressed as the mean (SEM). Correlations were expressed as the R value and the P value for the correlations was calculated using GraphPad prism version 5. The students T test was used to perform paired and unpaired analyses and p values <0.05 considered significant.

Chapter Three

The relationship between markers of ineffective erythropoiesis, oxidative damage and non transferrin bound Iron in children with thalassaemia syndromes

3.1 Introduction

Although the natural history of thalassaemia has been altered with the appropriate use of transfusion and chelation therapy as discussed in chapter one, patients still suffer serious complications from iron overload such as endocrinopathies and cardiac decompensation secondary to iron overload (Borgna-Pignatti, *et al* 1998, Borgna-Pignatti, *et al* 2004, Zurlo, *et al* 1989).

Oxidative damage secondary to iron overload is thought to be the key reason why fibrosis and cell death occur in organs such as the liver in thalassaemic patients. Markers of oxidative damage are increased in adults with iron overload (Cheng, *et al* 2005, Cighetti, *et al* 2002, Livrea, *et al* 1996). It is however, highly likely that a critical level of iron burden is necessary for iron to cause damage (Olivieri and Brittenham 1997) and it is quite probable that the duration of exposure to high iron burdens is also important (Gabutti and Piga 1996).

Patients with TI also may develop iron overload secondary to increased gastrointestinal iron absorption, although this is at a rate slower than that seen with blood transfusion. One can postulate that the driver of this increased absorption is

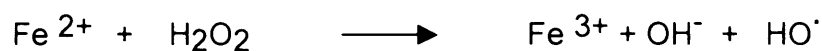
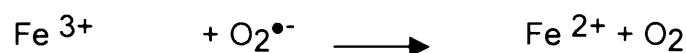
hypoxia/anaemia leading to increased erythropoietin with expansion of erythropoiesis. However, this does not explain why increased iron absorption is not seen in other anaemias where EPO levels are increased such as SCA. It is possible that the 'leakage' of non-transferrin iron into the plasma compartment secondary to the ineffective erythropoiesis (but less so with pure haemolysis) may play a role in increased iron absorption and oxidative damage in thalassaemia syndromes.

Iron exists in human sera as transferrin bound ferric iron, however once transferrin becomes fully saturated then free iron is found in the form of non-transferrin bound iron (NTBI). NTBI species can cause oxidative damage by generation of free radicals by the Haber- Weiss reaction (Halliwell and Gutteridge 1990). Reactive oxygen species such as superoxide can be derived from electron leakage from the mitochondrial electron transport chain.

Haber Weiss Reaction:



This reaction is catalysed by Iron in two steps (*Fenton reaction*):



Hydroxyl radicals (OH^\bullet) can cause lipid peroxidation (Aust, *et al* 1985, Halliwell and Gutteridge 1990), oxidation of cellular proteins, lipoproteins, nucleic acids, and other cellular constituents. Cell death results in fibrosis and loss of organ function and hence the complications of iron overload (Britton, *et al* 2002, Fleming and Bacon 2005, Houghlum, *et al* 1990, Junge, *et al* 2001).

There are a great many markers of oxidative damage that can be assessed but in this chapter we will examine the relationship between ineffective erythropoiesis, NTBI and malondialdehyde (MDA) and carbonyls.

3.1.1 Non transferrin bound iron (NTBI)

In the healthy physiological state in non-iron overloaded humans, iron is transported in the plasma as transferrin bound ferric iron. Transferrin is a key component of plasma and is the carrier protein that transports non-heme iron from sites of absorption or release to cells where it will be utilised. In health, transferrin is 20-35% saturated with iron and this is the only major form of non-heme iron in the plasma. In conditions of iron loading such as thalassaemia syndromes, transferrin rapidly becomes fully saturated and the plasma transport of additional reticuloendothelial derived iron cannot occur *via* the transferrin pathway. Under these conditions NTBI is formed.

NTBI was first identified by Hershko in thalassaemic patients in whose sera chelatable iron was measured in the presence of a fully saturated transferrin. When thalassaemic sera was added to normal sera the chelatable iron could no longer be detected (Hershko, *et al* 1978). NTBI was then found to occur in other iron loading conditions such as haemochromatosis and was linked to oxidative damage *in vitro* (Gutteridge, *et al* 1985). NTBI was also found to be present in patients with evidence of ineffective erythropoiesis but no iron overload (Corteletzi, *et al* 2000, Gafter-Gvili, *et al* 2004, Wickramasinghe, *et al* 1999).

There are two main principles applied for the methods that are in use currently for measurement of NTBI. The majority of methods are based on iron mobilisation, using shuttle molecules such as nitrilotriacetic Acid (NTA) followed by separation of chelated iron by ultrafiltration and detection using methods such as High performance Liquid

Chromatography (HPLC), atomic absorption, spectroscopy and inductive conductimetric plasma spectrometry (ICP) (Gosriwatana, *et al* 1999, Loreal, *et al* 2000). Other methods are based on the mobilisation and detection of iron without separation of it from plasma proteins; such as the use of flourescein probes which are sensitive to iron in an ELISA based method (Breuer and Cabantchik 2001) or measurement of redox- active iron using Bleomycin (von Bonsdorff, *et al* 2002).

There is significant variation in NTBI values obtained from the different methods used as shown by a recent study (Jacobs, *et al* 2005).

NTBI has been measured in this study using a method based on iron mobilisation on to NTA followed by separation of mobilised iron using ultrafiltration. The NTBI was then detected using HPLC using the method first described by Singh (Singh, *et al* 1990).

From the time that Hershko first described the presence of NTBI in the sera of thalassaemic patients, many studies have confirmed the presence of NTBI in patients with thalassaemia (al-Refaie, *et al* 1992a, Cappellini, *et al* 2000, Cighetti, *et al* 2002, De Luca, *et al* 1999, Grosse, *et al* 2005, Pootrakul, *et al* 2004, Wang, *et al* 1986) but of these studies only Wang and De Luca looked at NTBI in children with established transfusional iron overload. Pooktrakul and Cappellini looked at NTBI in TI and both found that levels were raised in the absence of transfusion.

No studies to date have looked at the appearance of NTBI in children very early on in the disorder to see if there is NTBI present, which may be related to ineffective erythropoiesis. In addition what happens to the NTBI secondary to ineffective erythropoiesis once transfusion support is initiated as well as whether there is a critical iron burden at which NTBI appears secondary to iron overload has not been studied. In this chapter the relationship between NTBI and the degree of transfusional load, as well as the effect of transfusion on NTBI in previously untransfused children will be discussed.

3.1.2 Markers of oxidative damage

Reactive oxygen species such as the hydroxyl radical and superoxide which can be generated in the presence of NTBI (Gutteridge, *et al* 1990), cause oxidation of cellular proteins, lipids etc. and result in the formation of stable end products that can be measured (Halliwell and Chirico 1993). Lipid peroxidation products are predominantly in the form of very reactive α,β -unsaturated aldehydes such as malondialdehyde (MDA), acrolein, and 4-hydroxy-2(E)-nonenal (HNE) (Picklo, *et al* 2002). These then go on to cause further oxidative damage in the form of protein alkylation, cross linking, mitochondrial dysfunction, altered nuclear signalling, inhibition of membrane transporters and further generation of reactive oxygen species (Del Rio, *et al* 2005). Oxidation of amino acids within proteins results in the conversion of amino side chains to intra protein aldehydes such as glutamic semialdehyde (from peroxidation of lysine and proline) and 2-amino-3-ketobutyric acid (from peroxidation of threonine) (Picklo, *et al* 2002). These intraprotein aldehydes (protein carbonyls) again can cause further oxidative damage as above (Reznick and Packer 1994).

3.1.2.1 Malondialdehyde (MDA)

MDA is one of the products of peroxidation of polyunsaturated fatty acids under conditions of oxidative stress. At neutral pH, MDA is of low reactivity but is still able to react with nucleic acid bases and form a number of toxic adducts such as pyrimido-[1,2- α] purine10(3H)-one deoxyribose which can cause sequence dependant frameshift mutations and base pair substitutions in mammalian cells. It can also form inter-strand

DNA cross-links as well as resulting in the formation of DNA- protein cross-links (Del Rio, *et al* 2005). These products damage the DNA and are 'genotoxic'. MDA like species are also thought to be involved in atherogenesis, by impairing the interaction between Low density Lipoproteins (LDL) and macrophages (Palinski, *et al* 1994). The most important effect of MDA however remains in its propensity for cross-linking collagen (Slatter, *et al* 2000). This may explain in part why fibrosis and cirrhosis are seen in patients with high iron burdens.

MDA exists in free and protein-bound forms. The free forms are not bound to any plasma protein and can be detected without any hydrolytic pre-treatment. The bound forms however are bound to different protein matrices and need to be liberated prior to measurement. Very little is known about the relative pathophysiological effects of the free and bound forms.

A number of methods are used for measuring MDA and the majority of them are based on derivation with thiobarbituric acid (TBA). The TBA forms an adduct with MDA which can then be measured using a spectrophotometric or a HPLC based method. Most methods involving TBA will measure total MDA (free and bound) because of the strong acidic and high temperature conditions required for the formation of the TBA-MDA adduct. The range of values for MDA has varied greatly in different studies and this variability is thought to be partly due to differences in the degree of matrix oxidation. Methods measuring TBA-MDA adducts without any refinement to overcome the matrix oxidation describe the final product as TBA Reactive substances (TBARS) and are essentially a global measure of lipid peroxidation.

This problem of matrix oxidation has been overcome to some extent by the addition of a step that precipitates the protein prior to oxidation with TBA such as using butylated hydroxytoluene (BHT) (Halliwell and Chirico 1993). A number of workers have also tried to overcome the problems of protein matrix oxidation by removing the protein

precipitation step and using fluorescence to detect MDA such as the method used by Del Rio (Del Rio, *et al* 2003). Other methods of measuring MDA are without the TBA derivation step and can detect both total MDA using strong acidic conditions or free MDA if the precipitation step is avoided. Only with gas Chromatography- Mass Spectroscopy (GC MS) are reaction conditions relatively mild, however this method still requires strong hydrolysis for the measurement of total MDA (Cighetti, *et al* 1999).

There are a number of studies looking at MDA in thalassaemia patients (Cappellini, *et al* 2000, Cighetti, *et al* 2002, Livrea, *et al* 1996, Meral, *et al* 2000, Naithani, *et al* 2006). Cighetti *et al* looked at a small number of adult beta thalassaemia using GC-MS and found levels to be raised significantly in TM. The study by Cappellini assessed adult non-transfused TI patients and found raised levels of MDA using a TBA based spectrophotometric assay. Livrea *et al* assessed MDA in both adults and children but the data was analysed as a single cohort and found that levels were raised. Only 2 studies (Meral, *et al* 2000, Naithani, *et al* 2006) analysed markers of lipid peroxidation in children specifically: Meral *et al* looked at TBARS and Naithani at MDA, both finding raised levels in these children. Naithani's study looked specifically at MDA and although the patient groups included children who had received very few transfusions, the data was not analysed for this group separately and therefore could not determine if there was a critical threshold of iron burden above which lipid peroxidation appears.

In this chapter, oxidative markers in children with thalassaemia disorders have been measured to see if damage starts early in life or whether this is a late effect occurring only after many years of excess iron loading.

3.1.2.2 Protein carbonyls

Protein carbonyls are generated when the side chain amino groups of amino acids within proteins are oxidised to form various aldehydes, resulting in the formation of further reactive species such as peroxides, bromamines and chloramines (Davies 2005). Oxidation of protein amino acid side chains can result in unfolding of the protein molecule and functional defects then may arise. These are generally not repairable. There is some evidence that chelation therapy is protective and may reduce the amount of oxidative damage secondary to iron (Cornejo, *et al* 2001, Reznick, *et al* 1992).

There are a number of methods for measuring protein carbonyls. The most well described is the method developed by Reznick and Packer which uses dinitrophenylhydrazine (DNP) to bind carbonyls which can then be detected calorimetrically using spectrophotometry (Reznick and Packer 1994). To overcome some of the limitations of the original method, this basic principle has been used to either detect or quantify protein carbonyls by a variety of techniques such as HPLC (Levine, *et al* 1994) western blot analysis (Shacter, *et al* 1994), or ELISA (Buss, *et al* 1997). Recently chemiluminescence which measure both protein and lipid carbonyls has also been used (Shnizer, *et al* 2003). In this study we used the original method as modified by Carmine (Carmine, *et al* 1995) and described in chapter 2.

There is little published data on carbonyls in patients with thalassaemia (Livrea, *et al* 1996, Ramenghi, *et al* 1989). It is unclear at which age oxidative damage starts and in the Livrea study the number of children was small and the results of the oxidative markers analysed as a single group finding that protein carbonyls were raised.

3.1.3 Markers of ineffective erythropoiesis

3.1.3.1 *Erythropoiesis in normal and in thalassaemia states:*

Erythrogenesis is dependent on a number of factors which when disrupted may result in ineffective erythropoiesis.

Iron is essential for normal erythropoiesis as discussed in chapter 1. Under normal physiological conditions, iron absorbed from the intestine or recycled from red cell turnover is transported from the enterocyte to the erythroblasts *via* transferrin. Transferrin binds to the transferrin receptor and iron is taken into the cell by receptor-mediated endocytosis. Once iron enters the cell, erythropoiesis can be completed with the formation of haemoglobin (Conrad, *et al* 1999).

In β thalassaemia syndromes, globin chain imbalance results in the precipitation of unpaired α globin in developing red cells in the bone marrow, causing ineffective erythropoiesis and early destruction of red cells in the circulation and the resultant haemolysis. The combined effects of ineffective erythropoiesis and haemolysis result in anaemia. The kidney detects the hypoxia resulting from anaemia; this then results in increased erythropoietin synthesis and secondary proliferation of erythropoiesis and erythropoietic tissue, which is reflected, by the increase in transferrin receptors.

The relationship between iron mediated oxidative damage and ineffective erythropoiesis is unclear. It has been shown in untransfused TI patients that NTBI is present at measurable levels in the serum (Cappellini, *et al* 2000, Cighetti, *et al* 2002). It is important to attempt to determine whether NTBI secondary to ineffective erythropoiesis plays a role in oxidative damage.

3.1.3.2 Erythropoietin (EPO)

The vital roles of erythropoietin (EPO) and the erythropoietin receptor (EPO-R), the role of stem cell factor (SCF) and its receptor c-Kit, as well as the intracellular signalling pathways that these receptors activate and their role on erythroid proliferation and survival have been all been clarified in recent years (Munugalavadla and Kapur 2005).

EPO is a 30,400 Dalton glycoprotein, which belongs to the class 1 cytokine family and initiates the signal for erythropoiesis by activating Janus Kinase 2 (JAK2). The activated JAK2 then is incorporated onto phosphorylated multiple tyrosine residues at various points on the EPO receptor (EPO-R). These then activate a number of intracytoplasmic signalling molecules that are involved in cell proliferation and cell survival (Munugalavadla and Kapur 2005).

EPO is produced by the peritubular cells in the kidney in adults and the hepatocytes in the foetus. It is secreted in response to hypoxia and anaemia (Fisher 2003). In normal healthy adults who are not anaemic, levels are found to vary between 1.6-34 mIU/ml and are raised in those patients with iron deficiency anaemia or haemolytic anaemia (Boyd and Lappin 1991, Haddy 1982, Roque, *et al* 2001) , Patients with anaemia secondary to renal insufficiency have low levels of EPO relative to the degree of anaemia (de Klerk, *et al* 1982, McGonigle, *et al* 1985, McGonigle, *et al* 1984) as do those patients with anaemia of chronic disease (Boyd and Lappin 1991, Gasche, *et al* 2004, Souweine, *et al* 1995).

Many studies on EPO have been undertaken on thalassaemia carriers and those with TI and TM (Camaschella, *et al* 1996, Cazzola, *et al* 1995, Dore, *et al* 1996, El-Nawawy, *et al* 2002, Galanello, *et al* 1994, Kalmanti, *et al* 1991, Nisli, *et al* 1997, Paritpokee, *et al* 2002). All of these studies have shown that EPO levels are raised in

transfused TM patients and in intermittently or non-transfused TI patients. The levels of EPO in TM patients tend to be raised despite 'hyper' transfusion regimes. Cazzola showed the EPO level varied according to the severity of the pre-transfusion anaemia and Kalmanti showed that in some patients EPO could be suppressed into the normal range. The raised EPO in TI patients is invariably due to the hypoxia induced by anaemia and studies where the HbF% has been used to help determine severity of tissue hypoxia such as those by Galanello and Chamaschella have shown that EPO levels tend to be higher in those patients with TI phenotypes associated with high HbF.

There has been much interest over the years in the potential use of EPO to help reduce the frequency of transfusions. Some workers have used recombinant human EPO (rHuEPO) either alone or with other modifiers of HbF production such as hydroxyurea (Chaidos, *et al* 2004, Makis, *et al* 2001, Olivieri, *et al* 1992, Rachmilewitz and Aker 1998, Rachmilewitz, *et al* 1991) with variable benefit. What has been noted is that higher doses of rHuEPO are required in thalassaemia patients and the increased erythroid drive is reflected by the increase in sTfR levels in those patients who respond (Chaidos, *et al* 2004, Dore, *et al* 1996).

3.1.3.3 Soluble transferrin receptor (sTfR)

Iron exists in the plasma in normal individuals as transferrin bound iron. Transferrin is the plasma protein responsible for transport of iron to various cells throughout the body that require iron. Transfer of iron occurs when transferrin binds to the transferrin receptors, which are membrane bound glycoproteins, allowing iron to enter into the cell by receptor mediated endocytosis of transferrin (Lamb, *et al* 1983, Yamashiro, *et al* 1984). The vast majority of transferrin receptors are found on the erythroid precursors of the bone marrow (80%) but significant numbers are also found

on the placenta and rapidly dividing cells in S phase (Beguin 2003). Although reticulocytes express transferrin receptors, mature red cells do not. The soluble transferrin receptor (sTfR) is a truncated monomer of the transferrin receptor lacking the initial 100 amino acids circulating in the serum as a complex with transferrin. sTfR are thought to originate from erythroblasts and to reflect bone marrow activity. The number of sTfR in the bone marrow is dependant both on the amount of iron available to cells and on the number of erythroid cells (Beutler, *et al* 2003). Iron deficiency leads to increased transferrin expression on developing erythroblasts and this is reflected by increased sTfR (Skikne, *et al* 1990). In hypoplastic erythropoiesis such as renal failure, aplastic anaemia or post chemotherapy sTfR is decreased, whereas sTfR is increased in conditions where there is an increased erythroid drive or erythropoietic expansion such as haemolytic anaemia, megaloblastic anaemia and polycythaemia (Beguin 2003, Ponchio, *et al* 1992, Roque, *et al* 2001). There is also a close relationship between ferrokinetic measurements of erythropoiesis and sTfR levels (Beguin, *et al* 1993, Cazzola, *et al* 1987). It is now a useful tool in distinguishing between anaemia of chronic disease and iron deficiency.

Many groups have looked at the relationship between sTfR and erythroid mass in thalassaemia (Camaschella, *et al* 1996, Cazzola, *et al* 1999, Cazzola, *et al* 1995, Dore, *et al* 1996, El-Nawawy, *et al* 2002, Galanello, *et al* 1994). All these studies have shown increased levels of sTfR in thalassaemia patients and that in patients with TM who follow hyper-transfusion regimes; these levels are suppressed into the normal range.

3.2 Study Rationale

It is unclear at which age or at what degree of iron loading, NTBI appears and its relationship to the advent of tissue damage is as yet undetermined. It is also unclear

when and at what iron burden oxidative damage starts: many thalassaemia patients develop evidence of pituitary dysfunction such as delayed puberty and primary amenorrhea at an early age, which is thought to be secondary to iron overload. Only a few investigators have looked into the relationship between iron burden and oxidative damage in thalassaemia syndromes.

It was decided to attempt to answer 3 specific questions with this study:

- I. Is there a difference in base-line parameters such as Hb, NTBI, EPO, sTfR, carbonyls and MDA in patients with thalassaemia disorders based on their demographics? These variables were therefore compared in patients treated in developed countries (Canada, UK) and patients treated in Sri Lanka.
- II. In patients with thalassaemia disorders, either transfused or non-transfused, is there a relationship between the markers of iron burden, ineffective erythropoiesis and oxidative damage and does this change with time?
- III. When does NTBI appear in thalassaemia patients, what is its relationship to transfusional iron load?

3.3 Patients and treatment centres

51 patients with TM or TI were enrolled into the study from three centres. Two centres were in countries where optimal treatment was readily available for both transfusion and chelation, funded either by the National Health Service or by Private Healthcare, the London Centre was University College London Hospitals (UCLH) and the other was Hospital for Sick Children, Toronto (HSC). Management at the London Centre was lead by Professor John Porter and in Toronto by Dr Melanie Kirby-Allen

(Sick Kids) and Dr Nancy Olivieri (Toronto General Hospital). These patients were recruited from November 1999 and the first samples were taken in March 2000. For this study the last patient samples to be included in the analysis were up to March 2004. There were 2 patients from London and 17 from HSC Toronto.

The third centre was in Kurunegala, Sri Lanka, where care was provided free of charge to the thalassaemic patients. Kurunegala State is in the South of Sri Lanka and was found to have the highest prevalence of thalassaemia. Prior to the start of this study, thalassaemia care had been undertaken on an ad hoc basis by the medical staff working in the State. Patients kept their own records and attendance for transfusion was often dependant on symptoms. There was no clear policy on the transfusion requirements of patients and very little distinction made between TI and TM patients by junior medical staff. It was decided by the senior clinician Dr Shanitmala de Silva to set up a collaboration with the Weatherall Institute in Oxford UK, and a centre was established in Kurunegala known as the National Thalassaemia Centre (NTCK) just prior to the start of the study, which looked after 300 patients with TM and 200 with E β thalassaemia (Premawardhena, *et al* 2004).

Mutational analysis had shown that a third of the thalassaemia population had E β thalassaemia and remainder of the population were either homozygous or compound heterozygotes for various β thalassaemia mutations and would have severe transfusion dependant anaemia. 24 different β mutations were identified and 4% of the population had triplicated or quadruplicated α gene defects.

A dedicated team of doctors comprising Drs Mahinda Arambepola, Ravindra Samaranayake, and Uditha Perera looked after the patients on a routine basis. Four monthly visits to the centre were carried out. Professor Sir David Weatherall from the Weatherall Institute in Oxford, UK, and Professor Nancy Oliveiri, Giulia Muraca and Laura Merson (research associates) from the University Health Network based at

Toronto General Hospital, Canada, comprised the team of international experts. The team of local and international experts saw patients and management was reviewed at these visits. Patients who had a TI phenotype (tolerant of anaemia with lower transfusion requirements) were weaned of transfusions slowly and those with significant iron overload were also chelated. Patients with TM phenotypes (thalassaemic facies, enlarged spleen, intolerant of significant anaemia) were started on regular transfusion support to maintain appropriate haemoglobin according to international criteria. A total of 37 patients were recruited from this site with samples dates between the October 2001 and November 2002 available for analysis.

At this time, patients who had been enrolled into the study had samples taken for NTBI and markers of ineffective erythropoiesis. The research associates collected samples for the assays from patients and ensured that all sample handling, freezing and shipping protocols were followed. The samples were stored at -20° C and transported in dry ice with the researchers to Oxford, UK and then couriered to UCLH making sure at all times that the cold chain was not broken.

A NIH Grant (DK55462) was awarded for this study and ethical approval was obtained for the studies from all the centres from which the patients were enrolled.

TM patients from the UCLH/HSC centres were those who required transfusion at 3 to 4 weekly intervals. TM patients from NTCK received transfusion support at 3 to 6 weekly intervals predominantly due to lack of awareness as to the need for regular transfusion. TI patients in the UCLH/HSC cohort were those who were transfusion independent or required <8 transfusions/annum. All of the UCLH/HSC TI (n=4) did become transfusion dependent and required transfusion support at 3 to 4 weekly intervals. In these patients data was included for the TI cohort only up to the time they required regular transfusion. Their results from post transfusion dependence were included in the TM cohort.

All patients with TM were recruited into the study before the age of 6 and all TI patients before the age of 9 yrs. UCLH/HSC patients comprised 23 patients (19 TM and 4 TI) and the NTCK cohort consisted of 28 patients (18 TM and 10 TI). 9 patients were in the study for 3 or more years.

Samples were collected 4 monthly into aluminised vacutainers and processed as described in chapter 2. Desferrioxamine was interrupted at least 48 hrs prior to sampling and the samples were collected with the pre-transfusion cross match and full blood count. Data was collected on transfusion and chelation.

Table 3.3.1.1: Patient characteristics for all groups

Patient characteristics	All patients	Thalassaemia major #	Thalassaemia intermedia *
Age years (mean \pm SEM)	3.95 (0.12)	4.62 (0.27)	4.8 (0.25)
Total number UCLH/HSC	23	19	4~
Total number NTCK	28	18	10^
Patients in study <1 yr	19	12	7
Patients in study >1<2 yrs	17	15	2
Patients in study >2< 3 yrs	5	3	2
Patients in study >3 <4 or more yrs	8	6	2
Patients in study >4 or more yrs	1	1	0

Legend: total of 51 patients in the study. 28 patients from NTCK and 23 patients from UCLH/HSC, 21 who were treated at Hospital for Sick Children, Toronto, Canada, and 2 at University College Hospital London.

#: All UCLH/HSC TM patients transfusion dependant 3 to 4 weekly. All NTCK TM patients transfusion dependant but transfused between 3 to 6 weekly

**TI: intermittently transfused or transfusion independent ~ all TI started transfusion (range 3.5 yrs to 7.5 yrs)*

^ 4 are transfusion independent 2 previously transfused, 2 never transfused

3.4 Methods

Samples were processed as described in chapter 2 and assays performed for NTBI, MDA, carbonyls, serum erythropoietin, and serum transferrin receptor.

Samples at UCLH were collected when the patients attended clinic at four monthly intervals, processed as per the protocols described in chapter 2 and frozen at -80 within an hour. The samples from HCS were handled similarly and were shipped every 4 months to UCLH via FedEx in dry ice. The samples are then stored at -80°C with the other samples for this study. NTBI and transferrin saturation was measured in all the samples within a month of the sample being taken from the patient (UCLH) or arrival at UCLH (NTCK and Toronto) and always within 3 months. The sTfR, EPO, Carbonyls and MDA measurements were undertaken on samples on a yearly basis using samples that had not been defrosted previously. Any samples that were found to be unintentionally defrosted on arrival were not used in the analysis.

3.5 Statistical analyses

Analysis was undertaken on the patients according to the type of thalassaemia. Further sub analysis was carried out in a number of cohorts as described below. The first analysis was on all patients from the 3 centres and is described in the text as 'All Patients'. The second analysis of data was according to centre i.e. UCLH/HSC compared to NTCK, to see if demographic and socioeconomic issues had an impact on levels of NTBI, EPO, sTfR, and markers of oxidative damage. The third set of sub analysis was to see if there is a relationship between individual markers of oxidative damage or ineffective erythropoiesis. Mean, median, standard deviation, standard error of the mean and students T test (unpaired arrays 2 tail and 2 sample equal variance)

were used to carry out the statistical analysis. All results in the tables are expressed as the mean (SEM). Correlations were carried out to see if there was a significant relationship between different parameters. P values for the correlations were calculated using GraphPad Prism version 5 software.

For section 3.8.3 all patients who were monitored prior to their first ever transfusion were separately analysed to look specifically at the appearance of NTBI and what happens to NTBI with the initiation of transfusion.

3.6 Overview of variables based on Underlying Disorder and Centres of treatment

3.6.1 Overview of results based on type of thalassaemia

In the first analysis undertaken, the key parameters measured were compared in all patients with TM or TI as defined above, independent of whether they were treated primarily in Sri Lanka, London or Toronto. The mean pre transfusion Hb, sTfR, and EPO are shown as markers of erythropoiesis. Markers of oxidative damage include protein carbonyls and MDA. Markers of iron metabolism include NTBI, transferrin saturation and serum ferritin.

Normal range for healthy normal adult controls in our lab were:

EPO: 6.27 mIU/ml \pm 2.99 mIU/ml; range 1.07 mIU/ml-10.5 mIU/ml; n=13

Carbonyls: 0.71nmol/mg \pm 0.08 nmol/mg; range 0.33-1.07 nmol/mg; n=12

MDA: 0.27 uM \pm 0.22uM; range 0.03-0.68uM; n=22

The sTfR range was 0.3 to 8.5mg/l for the kit used.

Table 3.6.1.1: Mean values for all parameters in all patients

Parameter (SEM)	TM (SEM) n=37#	TI (SEM) n=14*	p
Pre transfusion Hb g/dl	9.23 (0.12)	7.24 (0.29)	<0.0001
ferritin (ng/ml)	1274 (40.1)	491.5 (89)	<0.0001
Cumulative iron load from transfusion (g)	4.55 (0.25)	0.96 (0.31)	<0.0001
NTBI (umol/l)	3.92 (0.19)	2.12 (0.47)	<0.001
transferrin saturation	83.1 (1.54)	62.5 (4.01)	<0.0001
erythropoietin mIU/ml	422 (64.6)	667 (115.9)	ns
MDA (uM)	0.14 (0.02)	0.28 (0.06)	0.02
carbonyls (nmol/mg)	0.77 (0.02)	0.75 (0.06)	ns
soluble transferrin receptor (mg/l)	7.18 (0.4)	17.22 (1.24)	<0.0001

Legend:

: TM; all TM n=37 and + 4 TI (HSC) once they became transfusion dependant at 4 weekly intervals. Total samples analysed from TM or transfusion dependant TI = 222 samples

*: TI; all TI n=14 and -4 (HSC) once they became transfusion dependant. Results up to the time of first blood transfusion were included as TI and all results from time when they were transfused 4 weekly were included in the TM cohort. Total samples analysed for TI=40

ns: not significant

As can be seen, when all patients were analysed according to the underlying severity (TI versus TM) there were differences in the ferritin, NTBI, transferrin saturation, MDA and soluble transferrin receptors (sTfR).

One would expect the Hb to be lower in the TI and the ferritin would be higher in the TM because of the frequency of blood transfusion and subsequent iron loading. The sTfR was higher in the TI and as previously discussed sTfR is a reflection of the bone marrow erythroid mass, and the bone marrow activity is expected to be greater in patients who are more anaemic or less frequently transfused.

It is clear from Table 3.6.1.1 that NTBI and transferrin saturation is higher in TM patients than in TI. Further analysis in this chapter will seek to identify whether the presence of NTBI in TI patients is primarily a consequence of iron load (as suggested by raised serum ferritin), or a consequence of a higher degree of ineffective erythropoiesis as suggested by higher sTfR in TI patients.

In addition it is clear that carbonyls and MDA levels are normal in comparison to healthy adult controls and although there is a statistically significant difference in the MDA levels in the two forms of thalassaemia in young children, these are still within the normal range for our lab, accepting the fact that the MDA and carbonyl levels are not known for healthy non thalassaemic age matched children. This may suggest that lipid and protein peroxidation is a relatively late event developing after a period of prolonged exposure to higher iron burdens. In addition the fact that levels are within the normal range eliminates the possibility of poor sample handling as a factor. Previous workers (Naithani, *et al* 2006) have found raised levels on MDA's in children with thalassaemia but looked at children up to the age of 18 years with a mean ferritin of >3000ng/ml and average years of transfusion of 8.6 years. The children in the study by Naithani had had a much longer duration of exposure to iron and a higher iron burden compared to those patients in our study.

3.6.2 Overview of differences in variables according to patient treatment Centres

Before going on further to analyse the various factors affecting NTBI and other variables, it was important to see if there was a significant difference between centres. Tables 3.6.2.1 and Table 3.6.2.2 take the data in Table 3.6.1.1 and split it into patients

treated at UCLH/HSC (Table 3.6.2.1) and those treated at NTCK, Sri Lanka, (Table 3.6.2.2).

Table 3.6.2.1: Table of parameters for all UCLH/HSC treated patients

Parameter (SEM)	TM (n=19) #	TI (n=4) *	P
Pre transfusion Hb g/dl	9.4 (0.1)	8.0 (0.24)	0.0004
Ferritin (ng/ml)	1306 (41.4)	217.6 (21.5)	0.0001
Cumulative iron load (g)	5.07 (0.29)	0	<0.0001
NTBI (umol/l)	4.06 (0.21)	1.05 (0.26)	<0.0001
Transferrin saturation	81.1 (1.78)	49.2 (2.78)	<0.0001
Erythropoietin mIU/ml	219 (41.1)	513 (40.84)	ns
MDA (uM)	0.07 (0.01)	0.17 (0.01)	0.0200
Carbonyls (nmol/mg)	0.74 (0.06)	0.74 (0.06)	ns
Soluble transferrin receptor (mg/l)	6.26 (0.37)	19.1 (1.17)	<0.0001
Mean liver iron mg/g dw	8.7 (0.71)	ND	NA

Legend:

#: TM; (n=19 TM and +4 TI whose results are included post start of 4 weekly transfusions) all patients were on regular transfusion support at 3 -4 weekly intervals as discussed. Total samples analysed for TM=178

*: TI; All TI became transfusion dependant at 3 -4 weekly intervals. TI values are results for parameters prior to patient starting regular transfusion. Total samples analysed for TI =17

NS: not significant

ND: not done

NA not applicable

Chelation therapy was started on 13 of these children at the time of the analyses and the median dose was $2.4\text{g} \pm 2\text{g}$ of desferrioxamine per week. The 6 patients who had not started chelation at this time had not fulfilled the clinical criteria required for initiation of chelation at that time.

These results show that NTBI is present at relatively high levels in these children who have moderate to low iron burdens as reflected by the mean ferritin and liver iron.

The sTfR remains high in the TI patients and the markers of oxidative damage remain normal.

The TI patients from HSC were two sets of siblings. The first pair had codon 39 / IVS I-6, $\alpha\alpha/\alpha\alpha$, and started transfusion support aged at around the age of 3.5 and 4.2 years at HSC when the Hb fell below 7 g/dl and there was evidence of failure to thrive. The second pair had codon 8/9 / IVS II-1, $-\alpha^{3.7}/\alpha\alpha$, starting transfusion aged 6 and 7.5 years, they were lost to follow up in the year immediately prior to them starting transfusion due to the family moving away from the area, on their return to the clinic at HSC for annual review relevant transfusion history was collected, the reason for initiation of transfusion at the peripheral hospital remains unclear but is presumed that transfusion was initiated due to symptomatic anaemia. It is highly likely that the first pair of siblings were potentially TM but had some form of ameliorating factor resulting in the delayed development of symptomatic anaemia. The second pair of siblings had less globin chain imbalance due to the coinheritance of a single gene deletion alpha thalassaemia.

Interestingly these patients have detectable levels of NTBI in the presence of unsaturated transferrin prior to them starting transfusion support and also raised levels of sTfR. It is possible that the form of NTBI prior to initiation of transfusion and the development of significant iron loading in TI patients is different to that post transfusion or secondary to iron load.

Table 3.6.2.2: parameters in all NTCK treated patients

Parameter (SEM)	TM (n=18)#	TI (n= 10) *	p
Pre transfusion Hb g/dl	7.74 (0.33)	6.3 (0.37)	0.03
Ferritin (ng/ml)	1076 (72.6)	866 (111)	ns
Cumulative iron load (g)	2.43 (0.26)	1.66 (0.48)	ns
NTBI (umol/l)	3.61 (0.39)	3.39 (0.76)	ns
Transferrin saturation	90.2 (2.43)	78.1 (5.43)	ns
Erythropoietin mIU/ml	1253 (240)	791 (190)	ns
MDA (uM)	0.41 (0.06)	0.37 (0.10)	ns
Carbonyls (nmol/mg)	0.72 (0.06)	0.78 (0.08)	ns
Soluble transferrin receptor (mg/l)	12.83 (1.23)	16.02 (1.79)	ns

Legend:

#: TM; (n=18) all patients were on regular transfusion support at 3 to 6 weekly intervals as discussed. Total samples analysed for TM=42

*: TI; all patients were either transfused intermittently t >8 week intervals or had never been transfused (n=2). 4 had not received transfusion in the last year as per table 3.3.1.1.

All the TI patients (as defined as less than 8 transfusions /year) had HbE β thalassaemia but the exact beta chain mutation and the presence of any alpha chain defect was not available at the time of this analysis. The majority of those patients with TI phenotype were eventually weaned off transfusion. The NTBI, transferrin saturation, ferritin, MDA and sTfR are similar between the TM and TI patients at NTCK. It is noticeable that in NTCK the TI patients receive some transfusion and the TM patients receive less transfusion than in UCLH/HSC. Thus the difference between 'TI' and 'TM' is less in the NTCK patients. This may explain why NTBI is relatively raised in the TI patents in NTCK compared with those at UCLH/HSC. The lower transfusion regimen in 'TM' treated at NTCK is reflected in the lower mean Hb values, the higher sTfR (which is

nearly double that of UCLH/HSC patients) and the higher EPO levels (which are 6 times higher than the UCLH/HSC patients).

What can be seen clearly from this table is that the NTBI is present at significantly raised levels in both the TI and TM patients and that TI patients are similar to TM patients with respect to NTBI in Sri Lanka but not at UCLH/HSC. This may be due to the cumulative iron load but may also be related to the degree of erythropoietic stress as reflected by the greater degree of anaemia and raised levels of EPO and sTfR compared to the UCLH/HSC cohorts. As mentioned in section 3.3 transfusions were reduced and then stopped in patients with TI during the course of the study and poorly transfused TM patients had their frequency of transfusion increased. Although at the time of this interim analysis the cumulative iron burden is similar this should become dissimilar as more longitudinal data is obtained.

3.6.3 Discussion of differences between patients at NTCK and UCLH/HSC

There are significant differences between the cohorts in Tables 3.6.2.1 and 3.6.2.2. Cumulative iron load is higher in the TM patients from UCLH/HSC, the mean pre-transfusion Hb is lower in NTCK patients, and the MDA levels although still within in the normal range for our assay (range 0.03-0.68uM) (n=22) are significantly raised in the NTCK patients.

The cumulative iron load is highest in the TM patients from UCLH/HSC as they have been on optimal transfusion regimes while the TM from NTCK had lower cumulative iron as they were on less rigorous transfusion schedules. TI patients from NTCK had a higher cumulative iron compared to those from UCLH/HSC because these patients prior to the start of the study had been on intermittent transfusion regimes primarily due to the lack of knowledge amongst staff treating them at the time.

The TM NTCK cohorts were considerably more anaemic than those from UCLH/HSC with the mean pre-transfusion Hb about 2g below that seen in UCLH/HSC patients. Thus, as expected blood transfusion is associated with lower EPO values in the hyper-transfused UCLH/HSC patients (219 mIU/ml) than the low transfusion frequency patients in NTCK (1253 mIU/ml). This is also reflected in the levels of sTfR, which are twice as high in TM patients from NTCK (12.8mg/ml) compared to that in TM patients from UCLH/HSC (6.3mg/ml). The apparently small difference in pre-transfusion Hb (7.7g/dl) in NTCK TM patients compared with 9.4g/dl in UCLH/HSC patients has a two fold effect on stimulating erythroid mass and a five fold difference in erythropoietin.

Transfusion therapy can therefore account for the higher ferritin, NTBI and transferrin saturation. The degree of anaemia is quite similar in the two TI cohorts and hence the EPO and sTfR values are similar.

With respect to iron overload and iron mediated oxidative damage, serum ferritin and NTBI are similar in NTCK and other TM patients from UCLH/HSC. However, MDA appears to be higher in the NTCK patients ($p<0.001$), while the carbonyls are similar. This difference in MDA could reflect differences in chelation therapy as the cumulative transfusional iron burden is higher in the UCLH/HSC cohort and the mean MDA was much lower in the UCLH/HSC TM (0.07uM) compared to those from NTCK (0.41uM) ($p<0.001$). The TM patients from UCLH/HSC patients would be on regular and consistent chelation regimes whereas the NTCK patients were receiving less frequent chelation regimes reflected by the mean cumulative DFO of 189 g in UCLH/HSC versus 106g in the NTCK TM patients. In the TI patients the situation is very similar, none of the UCLH/HSC patients were transfused but 7 of the 9 TI from NTCK were transfused having a higher cumulative iron, with regards chelation this was given at a much lower frequency to the TI patients compared to the TM patients with only a mean cumulative DFO usage of 40g.

It is however difficult to be clear about the effect of other factors that may influence MDA levels in the cohort from NTCK such as malaria, and nutritional status. Recent studies in other countries where similar health problems exist have found low levels of antioxidant micronutrients and raised levels of oxidative markers in patients with TM and TI, (Al-Quobaili and Abou Asali 2004, Nasr, *et al* 2002) and E β thalassaemia (Laksmitawati, *et al* 2003).

Protein carbonylation appears to be a relatively late effect of iron overload and free radical damage and indeed Livrea's study in TM patients found a positive correlation between MDA and ferritin, and a positive trend with carbonyls (Livrea, *et al* 1996) and suggested that good adherence to chelation may help to reduce the appearance of these markers of oxidative damage. Our findings support the role of consistent chelation in reducing the levels of MDA.

The analysis of patients by demographics above, shows that the patients treated at NTCK initially were more anaemic and had a higher erythroid drive whilst those TM treated at UCLH/HSC had higher transfusion frequencies and a higher cumulative transfusion iron burden. Despite the higher iron load in UCLH/HSC TM patients, the levels of MDA were lower suggesting that the regular use of chelation therapy was modifying the levels. The NTBI was present in high levels in both cohorts of patients at both centres and the levels in TI patients prior to receiving transfusion therapy were raised at a young age before significant siderosis had developed (mean ferritin 217 ng/ml).

The relationship between markers of anaemia, ineffective erythropoiesis, iron burden and oxidative damage is suggested by the preliminary analyses but a more detailed analysis of individual parameters and their relationship to each other was conducted to confirm and support the preliminary findings.

3.7 The relationship between Ineffective erythropoiesis, oxidative damage and NTBI

3.7.1 Factors affecting serum Erythropoietin

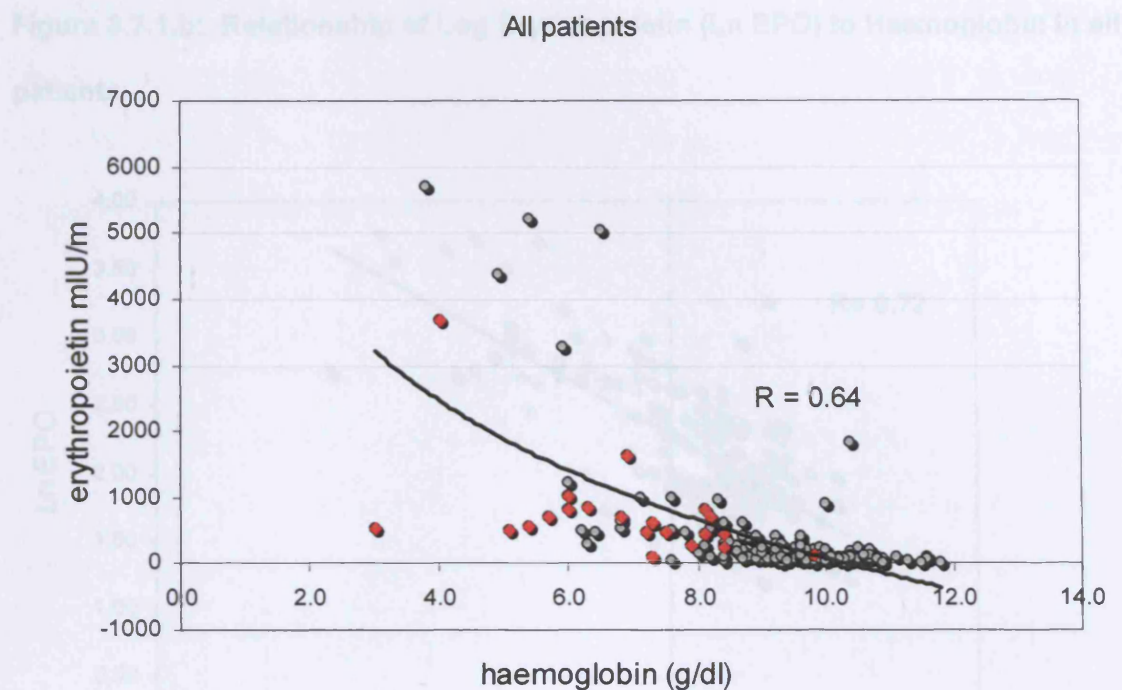
Serum EPO was performed as described in chapter 2 section 2.14.

The normal range for EPO in healthy controls for the EPO kit is 3.3 mIU/ml-16.6 mIU/ml and the normal range for healthy adult controls in our lab was 1.07 mIU/ml-10.5 mIU/ml.

3.7.1.1 Relationship between EPO and Hb

In tables 3.6.2.1 and 3.6.2.2 the variability of EPO by patient group was seen. It was apparent that in the TM patients EPO was not suppressed to near normal levels even in those patients who were on “optimal” transfusion regimes. Those who were under transfused as in the NTCK cohort the levels of EPO were higher than those of the TI patients treated at the same centre implying a greater hypoxia drive for erythropoiesis. Figure 3.7.1a shows the relationship between EPO and Hb in all patients in this study and Figure 3.7.1.b shows the relationship between the LnEPO and Hb.

Figure 3.7.1a: Haemoglobin versus erythropoietin in all patients



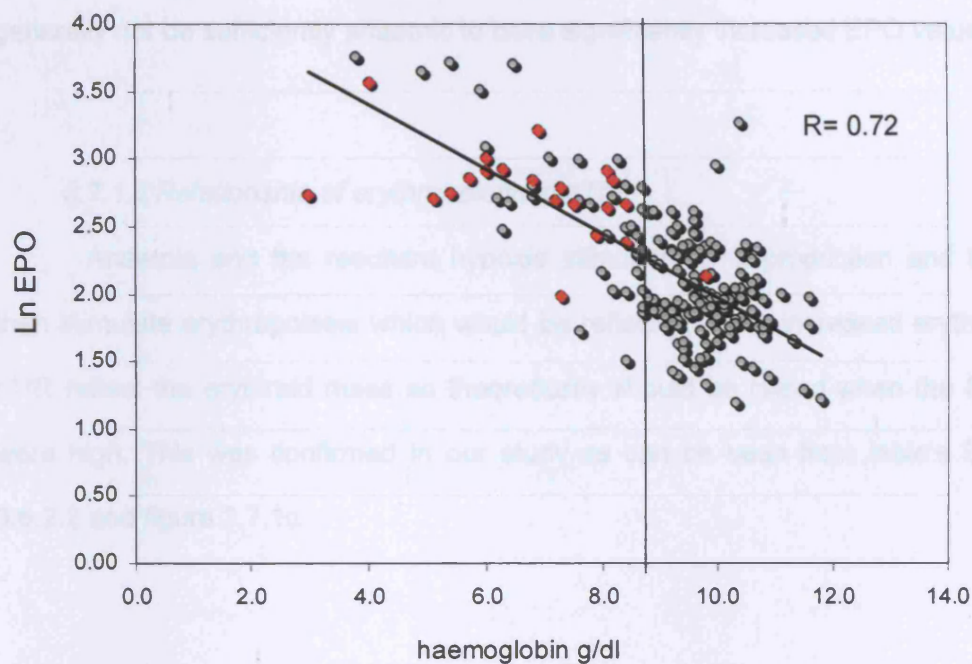
Legend: Erythropoietin for all patients plotted against the Hb. A good correlation is seen with Hb levels.

Total number of tests with simultaneous Hb and erythropoietin measurements $n=176$ $P<0.0001$

TI red diamonds; TM black circles

There is a good correlation between haemoglobin and erythropoietin in all patients ($R=0.64$ $p<0.0001$) and in the UCLH/BC dialysis patients ($R=0.63$ $p<0.0001$), but a much stronger correlation was seen with the more anaemic TM patients ($R=0.73$ $p<0.0001$) from NTGX. Our study shows a similar correlation to the hit as Cazzola et al (Cazzola, et al (1998).

Figure 3.7.1.b: Relationship of Log Erythropoietin (Ln EPO) to Haemoglobin in all patients



Legend: Log of erythropoietin plotted against Hb shows a good correlation in all patients Number of samples with Hb and erythropoietin measured on the same sample n=176. $P < 0.0001$

TI red diamonds; TM black circles

Line at 8.5g/dl: showing that majority of patients with TI in red have a significant anaemia associate with a higher LnEPO.

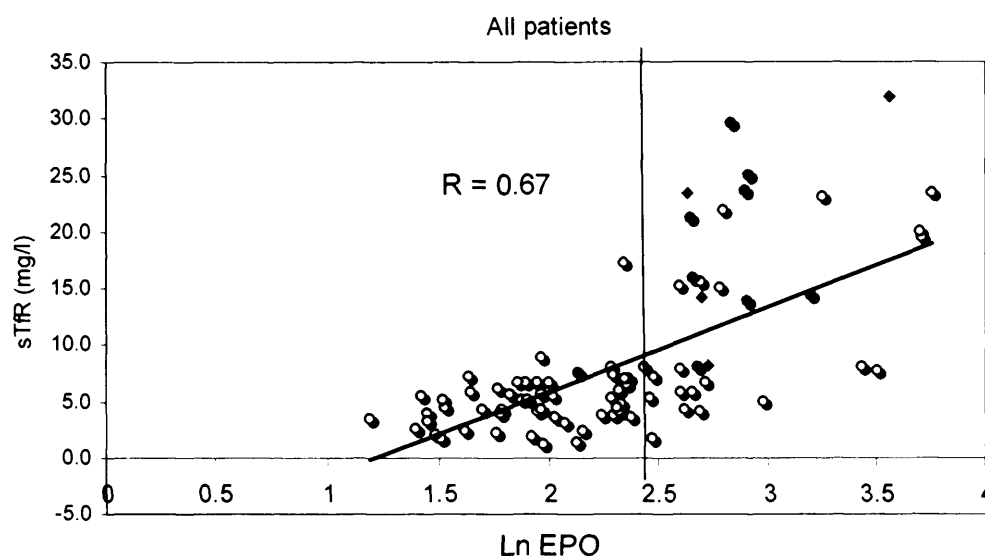
There is a good correlation between haemoglobin and erythropoietin in all patients ($R=0.64$ $p < 0.0001$) and in the UCLH/HSC thalassaemia patients ($R=0.53$ $p < 0.0001$) but a much stronger correlation was seen with the more anaemic TM patients ($R=0.73$ $p < 0.0001$) from NTCK. Our study shows a similar correlation to the Hb as Cazzola et al (Cazzola, et al 1999).

A number of studies have shown a relationship between the sTfR and erythropoietin levels (Cazzola, *et al* 1995, Galanello, *et al* 1994, Roque, *et al* 2001) and this direct relationship was also seen in our patients. If figure.3.7.1b is examined; 'abnormal' EPO values are confined (with the exception of two outliers) to patients with Hb values of less than 8.5g/l. This means that patients transfused in UCLH/HSC will generally not be sufficiently anaemic to have significantly increased EPO values.

3.7.1.2 Relationship of erythropoietin to sTfR

Anaemia and the resultant hypoxia stimulate EPO production and this should then stimulate erythropoiesis which would be reflected by an increased erythroid mass. sTfR reflect the erythroid mass so theoretically should be raised when the EPO levels were high. This was confirmed in our study as can be seen from table's 3.6.2.1 and 3.6.2.2 and figure 3.7.1c.

Figure 3.7.1.c Relationship between EPO and sTfR in all patients



Legend: The sTfR plotted against Log Epo in all patients who had Hb, EPO and sTfR measured simultaneously (samples analysed $n=85$) $P<0.0001$

TI red diamonds; TM black circles

Erythropoietin also had a good correlation with sTfR ($R=0.67$, $P<0.0001$). Higher sTfR levels are found in patients with higher erythropoietin levels and these tend to be associated with lower haemoglobins. It can be seen from figure 3.7.1c that 'abnormal' sTfRs are confined to patients with LnEPO values > 2.4 . From inspection of figure 3.7.1c, it can be seen that LnEPO of 2.4 is roughly equivalent to a Hb value of 8.8g/dl. Thus a Hb of $<8.5\text{g/dl}$ increases the chance of both an increase in EPO and an expansion of the erythroid mass, as measured by sTfR.

Interestingly the use of high intensity transfusion regimes appears to push the sTfR into the normal range but the EPO tends to remain raised (table 3.6.2.1)

3.7.1.3 Relationship of erythropoietin to Markers of oxidative damage

There appears to be a correlation between EPO and protein carbonyls ($R=0.33$ and $P=0.0003$; $n=82$) but with a wide scatter, suggesting that anaemia and ineffective erythropoiesis may result in increased formation of protein carbonyls, however this is unlikely to be the case as the protein carbonyls in group analysis showed little variation despite significantly different EPO levels (tables 3.6.2.1 and 3.6.2.2). There was no correlation of MDA with EPO and $R=0.04$; $p=0.80$; $n=81$.

3.7.1.4 Relationship of EPO with ferritin and NTBI

There was a weak correlation seen between LnEPO and ferritin ($R=0.27$; $P=0.004$; $n=104$) but with a wide scatter. This could be due to the fact that the more anaemic patients would have a lower ferritin and a higher EPO due to the less frequent transfusions. There was no correlation between LnEPO and NTBI ($R=0.05$; $p=0.87$; $n=83$).

EPO has a good relationship with STfR and Hb as seen in previous studies by other groups but is not affected by NTBI and does not appear to have a significant relationship with markers of oxidative damage or iron overload.

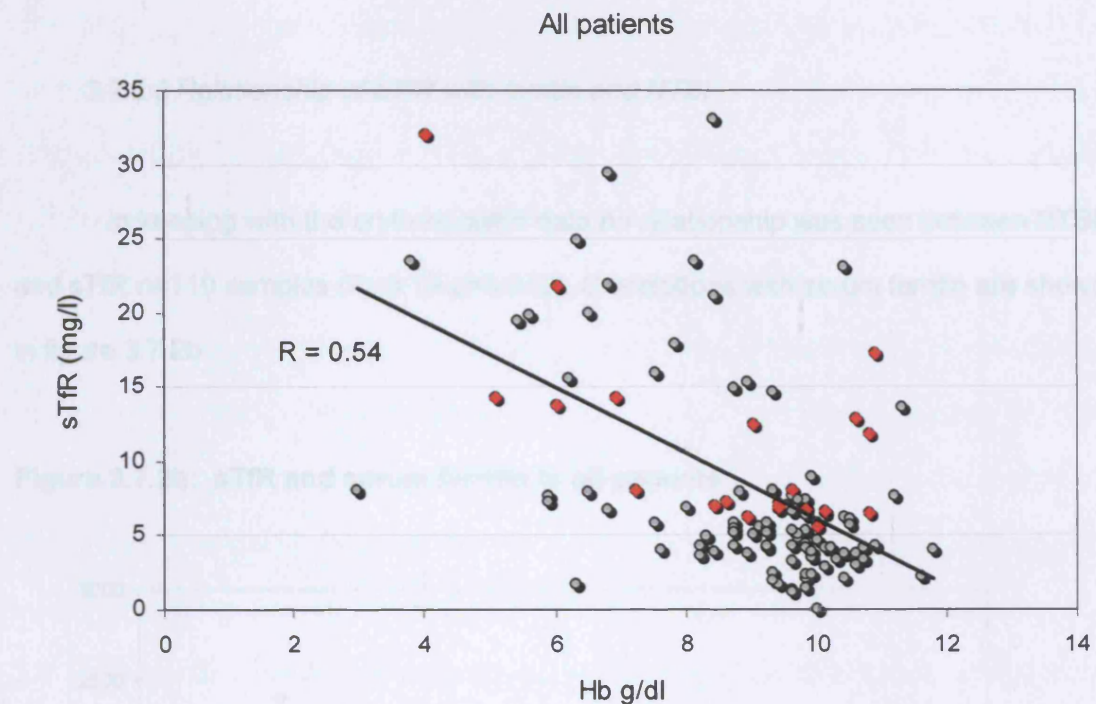
3.7.2 Variables affecting Transferrin receptors (sTfR)

3.7.2.1 sTfR and Haemoglobin

Transferrin receptors have been shown to increase in response to anaemia in TI patients by Cazzola (Cazzola, *et al* 1995) and rose significantly with haemoglobin levels below 9g/dl. In this section we examine the relationship between Hb and sTfR in all patients and to confirm the relationship shown between EPO and sTfR. The normal range of transferrin receptors is 0.3-8.5 mg/l.

With transfusion support, the mean sTfR level for TM comes into the normal range as shown in table 3.6.2.1. Levels however remain high for TI patients on intermittent regimes and also for the TM patients from NTCK who are subjected to a low transfusion regime as shown in table 3.6.2.2. The relationship between sTfR and Hb for all patients is shown in Figure 3.7.2a

Figure 3.7.2a: Relationship of sTfR to haemoglobin



*Legend: sTfR in all patients who has simultaneous samples for Hb (n=117) R 0.54 P<0.0001
TI red diamonds; TM black circles*

There was a good correlation and $P<0.0001$ but this cannot be confirmed for the more anaemic TI patients as the numbers are too small. As this study continues to recruit the relationship will become clearer.

The relationship between EPO and sTfR has already been described in figure 3.7.1c.

3.7.2.2 Relationship of sTfR and markers of oxidative damage

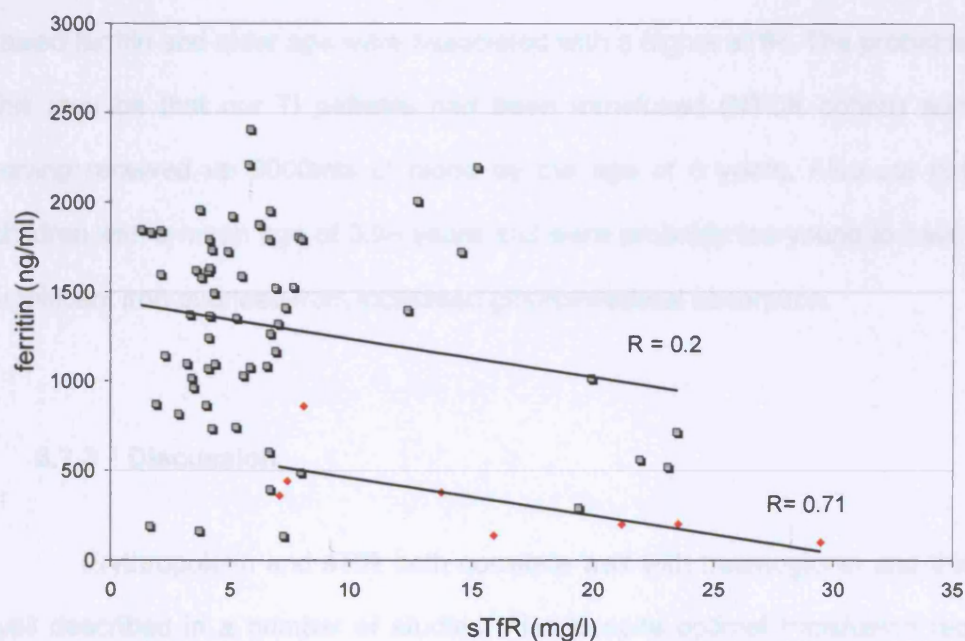
A weak correlation was seen between carbonyls and sTfR as there was a wide scatter ($R=0.25$; $p=0.02$ $n=83$). Again it is difficult to say if this is truly significant and

more data will need to be analysed to confirm these results. It is also imperative that a normal range for protein carbonyls in children is ascertained. There was no correlation with MDA ($R=0.17$; $p=0.11$ $n=82$) and the level of sTfR.

3.7.2.3 Relationship of sTfR with ferritin and NTBI

In keeping with the erythropoietin data no relationship was seen between NTBI and sTfR $n=110$ samples ($R=0.19$ $p=0.049$). Correlations with serum ferritin are shown in figure 3.7.2b

Figure 3.7.2b: sTfR and serum ferritin in all patients



Legend: sTfR in those patients with simultaneous ferritin measurements available ($n=70$)

$R=0.41$ $p=0.0005$ for all patients subanalysis. TI $R=0.71$; $P=0.15$ and TM $R=0.20$ $P=0.11$

TI= red diamonds and red line ; TM= black circles and black line

There was a correlation of sTfR with ferritin ($R=0.41$; $P=0.0005$; $n=70$) overall for all patients. But on subgroup analysis this relationship did not hold. Interestingly Cazzola's group (Cazzola, *et al* 1999) had shown a positive correlation between ferritin and sTfR as well as a relationship between sTfR multiplied by age and ferritin on multivariate analysis in patients with TI, in our patients there was no significant relationship as the numbers were too small for a significant P value. All of the patients in Cazzola's study were a mixture of adults and children but were non transfused (15/30) or had received less than 12 units of blood in their life time (15/30).

In our transfused patients, the lack of a relationship between sTfR and ferritin is likely to result from repeated transfusions decreasing erythroid expansion, but at the same time increasing iron overload. In the TI patients we found that the higher sTfR levels were associated with lower ferritin levels contrary to the findings of Cazzola where raised ferritin and older age were associated with a higher sTfR. The probable reason for this may be that our TI patients had been transfused (NTCK cohort) some of them having received up 8000mls of blood by the age of 8 years. Also our patients were children with a mean age of 3.95 years and were probably too young to have developed significant iron overload from increased gastrointestinal absorption.

3.7.3 Discussion

Erythropoietin and sTfR both correlate well with haemoglobin and this has been well described in a number of studies. Also despite optimal transfusion regimes EPO levels are not suppressed to normal levels in TM patients. STfR is however closely related to the degree of ineffective erythropoiesis and was suppressed into the normal range in our optimally transfused TM patients but not suppressed in the TI patients or the under transfused NTCK TM patients. A relationship was observed between EPO

and ferritin presumably because the more anaemic patients have less iron loading due to lower transfusion rates. No relationship however was seen between sTfR and ferritin and there appears to be a suggestion of a relationship between sTfR and carbonyls and between EPO and carbonyls although this will need to be confirmed with larger numbers.

The relationship of NTBI with anaemia will be discussed later in section 3.8

Iron overload will lead to lipid and protein peroxidation as discussed in section 3.1. The time of appearance of markers of oxidative damage and their relationship to iron burden and NTBI is unclear. Although a number of small studies have assessed these parameters in both young and older patients the data has not been analysed in very young patients separately to identify when these parameters appear and what their relationship may be to the patient's clinical status. As has already been noted from the preliminary analyses in section 3.6.3 and table's 3.6.2.1 and 3.6.2.2 the MDA levels are significantly higher in those patients from NTCK.

3.7.4 Variables affecting Malondialdehyde (MDA)

MDA levels in the normal adult control population used for this study in our laboratory was 0.27 ± 0.22 uM (range 0.03-0.68uM) (n=22). The patients who were treated at NTCK were significantly more anaemic then those at UCLH/HSC. An analysis was undertaken of al the patients to see if there was any relationship between MDA and anaemia and markers of ineffective erythropoiesis.

3.7.4.1 MDA and its relationship to Anaemia

There was a weak correlation seen between anaemia and MDA (n=192 R=0.15; p=0.03). As can be seen from tables 3.6.2.1 and 3.6.2.2 MDA levels were higher in the more anaemic patients. The significance of this is uncertain because the MDA values are in the normal range for adults and hence no real conclusions can be made from this analysis. It is important that control MDA results are obtained from normal non thalassaemic children to ascertain the significance of these results. As discussed above in section 3.7.2 and 3.7.3, no correlation was seen with markers of ineffective erythropoiesis.

3.7.4.2 MDA and its relationship to NTBI, ferritin and carbonyls

No relationship was seen between MDA and NTBI (R=<0.01; P= 0.81;n=187) similar to the finding in Livrea's study. However Cighetti (Cighetti, *et al* 2002) more recently found a relationship with NTBI in adult thalassaemia patients using GC-MS to measure free and total MDA. The reason for the difference may be that different methods were used to quantify MDA and our population of patients were much younger and had MDA levels within the normal range.

We saw no correlation between MDA and ferritin (R=0.01; P=0.88; n=111) in our study. Cighetti noted a negative correlation with ferritin (R= - 0.3) whilst Livrea found a positive correlation with ferritin (R=0.41). Ferritin is affected by the ascorbate status of patients and in Livrea's study there was a 44% reduction in ascorbate levels in patients. Cighetti did not measure individual antioxidants but assessed the total peroxyl radical-trapping antioxidant parameter (TRAP), which was found to be within the normal range.

There does not appear to be a clear relationship between MDA and ferritin in our study in young patients.

There appears to be a weak correlation between MDA and carbonyls ($R=0.24$; $p=0.006$; $n=134$). As both are markers of oxidative damage and both are in the normal range it not possible to draw any meaningful conclusions.

3.7.5 Variables affecting Protein Carbonyls

Carbonyls levels for the normal adult control population used in this study were 0.71 ± 0.27 nmol/mg (range 0.33-1.09) $n=12$. Only a few studies have looked at protein carbonyls in thalassaemia patients with the definitive study the one done by Livrea which showed raised levels of protein carbonyls in adult TM patients.

3.7.5.1 Relationship of carbonyls to anaemia

As has been shown in tables 3.6.2.1 and 3.6.2.2 protein carbonyls do not appear to alter in patients when dividing them according to the centres or when separating them according to the type of anaemia. There was a relationship with Hb ($R=0.28$; $p=0.009$; $n=133$) with higher carbonyl levels being seen with higher haemoglobin levels. There is however a wide distribution on the graph. As has been previously discussed there is a relationship between carbonyls and erythropoietin and sTfR.

3.7.5.2 Carbonyls and its relationship to NTBI, ferritin and MDA

There was no relationship between carbonyls and ferritin ($R=0.09$; $P=0.42$; $n=77$) or carbonyls and NTBI ($R=0.08$; $P=0.34$; $n=133$) in the patients as a group or in any of

the subgroups. No relationship was seen with MDA as mentioned in 3.7.5.2. This is probably because the carbonyls were not raised in our patients.

No relationship was seen between MDA, carbonyls and transfusion/ cumulative iron load.

3.7.6 Discussion

Our results show that carbonyls and MDA were not raised in thalassaemic children who were part of this study. In addition there was no relationship with ferritin or NTBI, contrary to the prior studies as discussed earlier where correlations had been found with ferritin (Livrea, *et al* 1996) and NTBI (Cighetti, *et al* 2002). In particular the conflicting results found with correlations between MDA and ferritin by Cighetti and Livrea and the lack of correlation with NTBI shown by Livrea raise important questions. On reviewing the data by Livrea the plot of the MDA versus ferritin shows a wide distribution and the $R = 0.41$; $n=42$) shows some correlation but this is still relatively poor particularly in view of the distribution. Cighetti's data again shows a very wide distribution and shows a negative correlation of free MDA with ferritin ($R=-0.3$; $n=21$). Both of these studies were carried out in a mainly adult population of thalassaemia patients. The role of chelation therapy is also extremely important in oxidative damage as this removes free iron that can initiate hydroxyl radical damage and in both studies patients were chelated with DFO so this could not be a cause for the difference.

Cighetti et al 2002; Eur J Clin Invest, 32 suppl. 1,55-60

Livrea et al 1996, Blood, 88, 3608-3614

It has been well described that when a DFO infusion is initiated the NTBI decreases to very low levels and once the infusion stops NTBI again becomes detectable in the blood (Porter, *et al* 1996). It may be that a consistent and prolonged exposure to Iron in the form of NTBI is required for free radical damage to occur. If a

child were regularly adhering to chelation on 5 to 6 nights a week this would result in relatively low exposure to high levels of NTBI. Indeed the cohort of children treated at UCLH/HSC is the one expected to have minimal iron overload associated complications as supported by data from Borgna-Pignatti (Borgna-Pignatti, *et al* 2004). The children from NTCK were by and large being transfused at variable intervals at the time of this analysis as were most of the TI patients who were being weaned of transfusion regimes. Chelation was also variable but most NTCK children had received some and the mean DFO given in TM was 106g and TI was 40g.

Other factors that may affect the results would be poor sample handling and storage. However, in the presence of NTBI plasma proteins and lipids would result in more oxidation occurring and higher levels of MDA and carbonyls. This was not noted in our patients who had levels within the normal range.

It can be concluded that in children with thalassaemia who are adequately treated with chelation therapy markers of oxidative damage such as MDA and carbonyls tend to remain low.

This is a longitudinal study and it will be interesting to see what happens to these markers as the children enter adolescence and if they develop any significant complications of iron overload. What is also clear is the NTBI and ferritin in this cohort of young children shows no correlation with markers of ineffective erythropoiesis or oxidative damage. However, NTBI is present at high levels in those patients who have not previously been transfused implying a relationship with ineffective erythropoiesis.

3.8 Factors affecting Non Transferrin Bound Iron (NTBI)

The relationship of NTBI with transfusion and anaemia is an intriguing one. Many patients with TI have detectable NTBI even when they are not transfusion dependant (Cappellini, *et al* 2000, Cighetti, *et al* 2002, Pootrakul, *et al* 2003). The question as to whether this reflects iron overload, ineffective erythropoiesis or increased iron absorption from the gastrointestinal tract is an important one. In addition quite frequently the serum transferrin is not fully saturated when NTBI s present (Gosriwatana, *et al* 1999) .

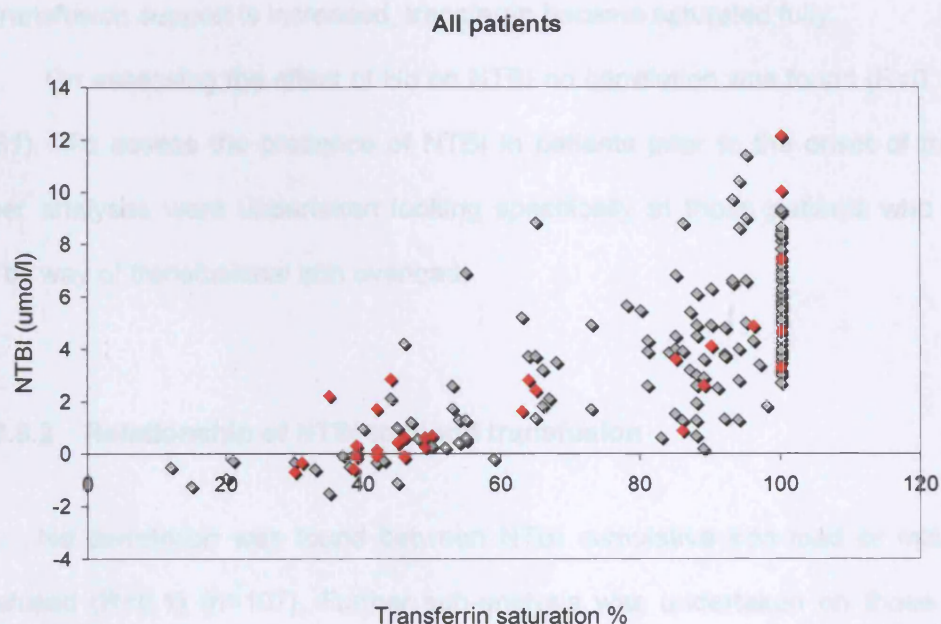
In section 3.8.1 data will be presented on all the patients for NTBI and transferrin saturation and its relationship to anaemia discussed. Then a more detailed assessment of patients who initiated transfusion therapy after recruitment into this study, and where samples were available, pre and post the first transfusion episode will be undertaken. In addition an assessment of NTBI and other markers will be carried out in those patients who had less than 1000mls of blood.

Of the 51 patients only 7 started transfusion whilst in the study. All of the NTCK patients were either already on a transfusion regime or did not required a transfusion at the time of this analysis.

3.8.1 NTBI and its relationship with transferrin saturation and anaemia

NTBI is thought to appear once transferrin is fully saturated; however it appears that even when transferrin is not saturated NTBI may be present at high levels as shown in figure 3.8.1a

Figure 3.8.1a: Transferrin saturation and NTBI for all patients



Legend: NTBI and transferrin saturation measured in all patients showing the presence of unsaturated transferrin in the presence of NTBI (n=181).

red diamonds: TI ; black circle TM

As can be seen NTBI is present in the presence of unsaturated transferrin. However no NTBI is found in patients with transferrin saturation less than one third saturated (Figure 3.8.1a). The presence of NTBI even in the absence of completely saturated transferrin has previously been identified by (Gosriwatana, *et al* 1999, Gutteridge, *et al* 1985) in haemochromatosis patients. These patients develop iron overload due to increased iron absorption from the gastrointestinal tract. Thalassaemia patients develop iron overload due to both increased gastrointestinal iron absorption (TI) and secondary to blood transfusions (TM). It is possible that there are different isoforms of NTBI some of which are transferrin accessible and some of which are not. The exact

nature of NTBI may thus depend on the mechanism and degree of iron loading. Interestingly non-transfused TM patients had NTBI and unsaturated transferrin but as the transfusion support is increased, transferrin became saturated fully.

On assessing the effect of Hb on NTBI no correlation was found ($R=0.1$; $P=0.17$ $n=181$). To assess the presence of NTBI in patients prior to the onset of transfusion further analyses were undertaken looking specifically at those patients who had very little by way of transfusional iron overload.

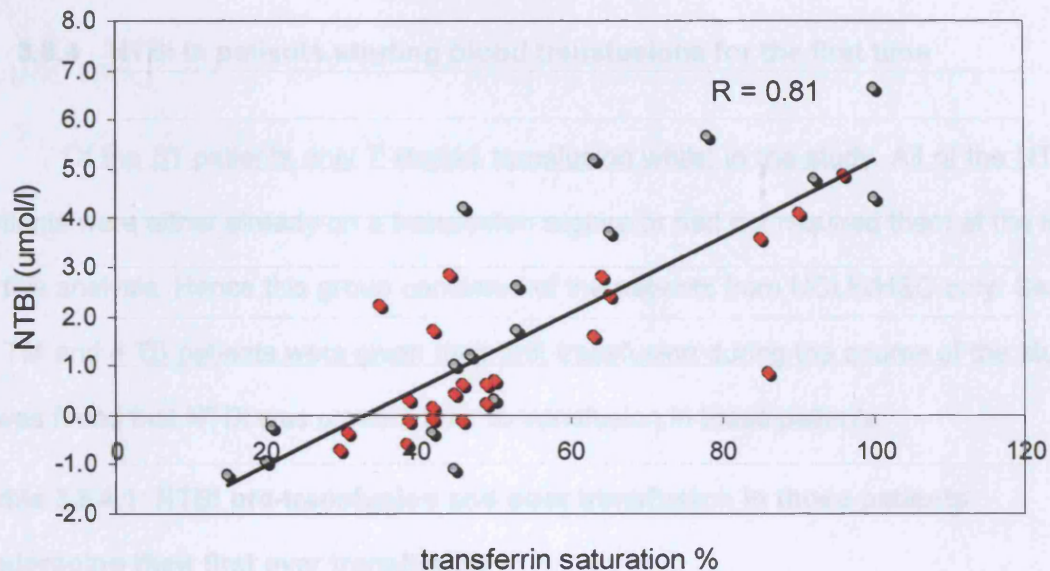
3.8.2 Relationship of NTBI to blood transfusion

No correlation was found between NTBI cumulative iron load or mls of blood transfused ($R=0.1$) ($n=197$). Further sub-analysis was undertaken on those who had received less than 1000ml of blood.

3.8.3 Patients transfused less than 1000mls

Fifteen patients (5 TM, 10 TI) had been transfused less than 1000mls and by virtue of this were not on chelation therapy and were thought to have a low iron burden. Of the patients who were transfused less than 1000mls a very good correlation was found between NTBI and transferrin saturation as shown in figure 3.8.3a.

Figure 3.8.3.a: Transferrin saturation for 15 patients transfused less than 1000ml of blood



Legend: 15 patients who had received less than 1000ml of blood. (Sample $n=42$) shows a good correlation of NTBI with transferrin saturation, $P<0.0001$

Red diamonds: TI ; Black circles TM

Again it can be seen that there are patients with high levels of NTBI and unsaturated transferrin. The patients with TM tended to have higher transferrin saturations in this small group of patients who had only recently initiated transfusions.

Correlations were undertaken on these patients to see if there was a relationship between anaemia and NTBI or markers of oxidative damage.

No correlation was found between NTBI and volume of blood transfused (R=0.14; P=0.35; n=47), haemoglobin (R=0.21; P=0.15; n=45), MDA (R=0.03; P=0.88; n=35) or carbonyls (R=0.24; P=0.22; n=28).

3.8.4 NTBI in patients starting blood transfusions for the first time

Of the 51 patients only 7 started transfusion whilst in the study. All of the NTCK patients were either already on a transfusion regime or had not required them at the time of this analysis. Hence this group consisted of the patients from UCLH/HSC only. Seven (3 TM and 4 TI) patients were given their first transfusion during the course of the study. It was found that NTBI was present prior to transfusion in these patients.

Table 3.8.4.1: NTBI pre-transfusion and post transfusion in those patients undergoing their first ever transfusion

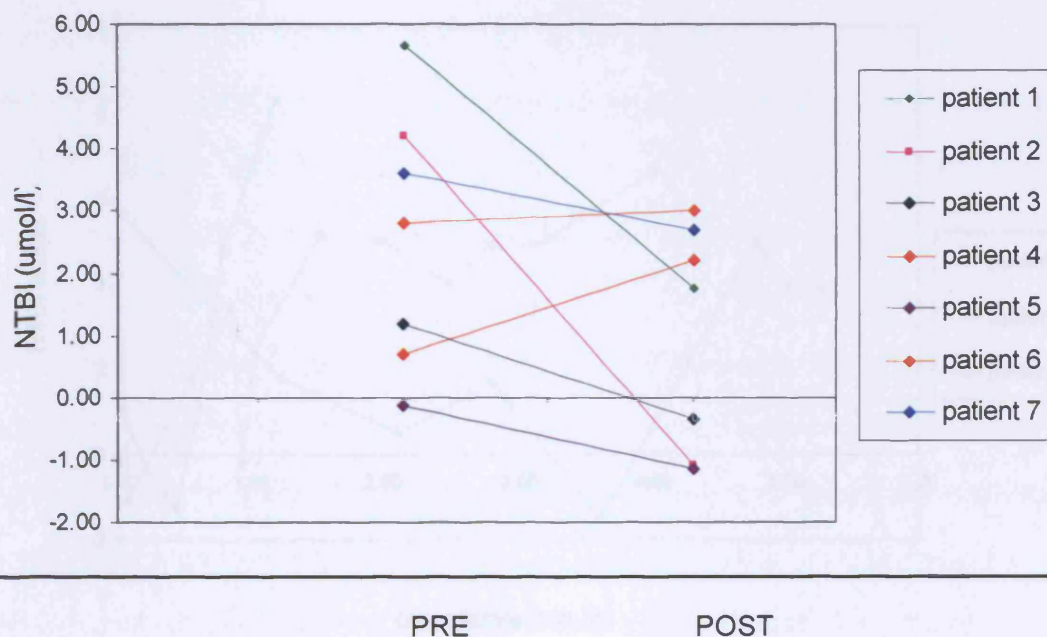
Patient	Diagnosis	Pre transfusion NTBI (umol/l)	Post transfusion NTBI (umol/l)	Interval between sample (months)
Patient 1#	TM	5.65	1.77	3.22
Patient 2*	TM	4.20	-1.10	0.17
Patient 3*	TM	1.20	-0.35	2.07
Patient 4	TI	0.70	2.20	3.81
Patient 5	TI	-0.12	-1.16	3.68
Patient 6^	TI	2.80	3.00	15.8
Patient 7^	TI	3.60	2.70	15.8

Legend: # Toronto patient Pre transfusion Hb was 5.4g/dl. Post transfusion sample 3 months later post 1500 mls of blood.

*: Patients treated at UCLH. Patient 2; Hb 2.4g/dl at presentation post Hb was 6 days later when Hb 10.2. Patient 3; Hb 5.4 g/dl post Hb was 2 months later post 250 ml blood.

^: Siblings with TI. Started transfusion due to failure to thrive. Baseline NTBI level prior to leaving Toronto. Post NTBI result is after <1500 ml of blood had been transfused. Only started Transfusion 3 months prior to post sample.

Figure 3.8.4a: NTBI pre-transfusion and post transfusion in those patients undergoing their first ever transfusion

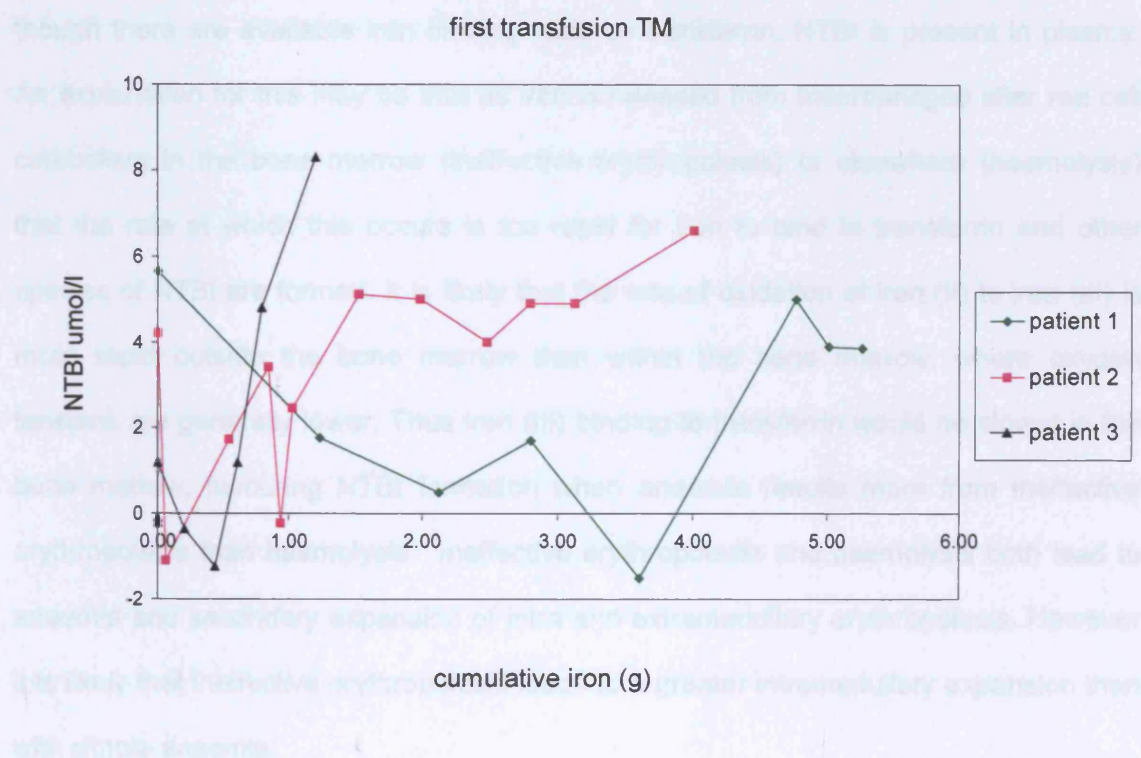


Legend: Same patients as in table 3.8.4.1. Overall the post transfusion NTBI levels are lower (mean 1.01umol/l) than those from pre transfusion (2.58 umol/l).

Once transfusion was administered NTBI generally fell to lower levels in both TI and TM. That NTBI is present at high levels when patients are very anaemic prior to their first transfusion and is present in TI patients at modest degrees of anaemia is an interesting observation. It was important to see what happened to NTBI with increasing

iron load. Three of the TI patients all had NTBI at first transfusion and this did not alter, one of the TI patients (patient 5) did not have any NTBI at the start of transfusions. In view of this the TM patients were analysed because they all had profound anaemia prior to first transfusion.

Figure 3.8.4b: NTBI and cumulative iron load in patients with TM who had their first transfusion as part of this study



*Legend: cumulative iron load versus NTBI in TM patients undergoing first ever blood transfusion
Cumulative iron load as calculated by multiplying the mls of blood transfused with the haematocrit as per the formula described by Porter (Porter 2001)*

As can be seen from figure 3.8.4b NTBI is present prior to transfusion and then decreases with the first transfusion as the anaemia is corrected. Then NTBI reappears

and fluctuates. As transfusion iron load increases NTBI increases again. The early NTBI may be due to ineffective erythropoiesis and increased GI iron absorption similar to that seen in TI patients. The levels of EPO and sTfR both fell post transfusion in keeping with what is expected to happen as the ineffective erythropoietic drive is suppressed.

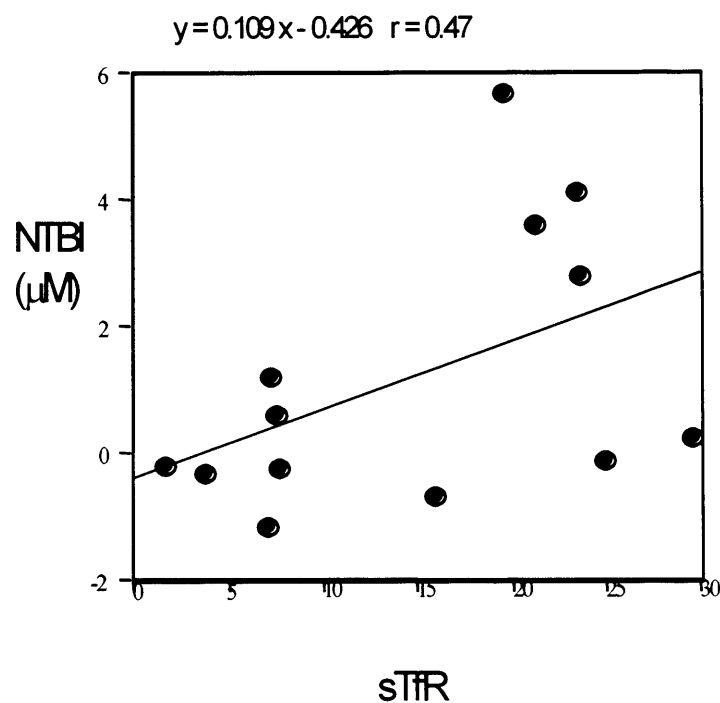
3.8.5 Relationship of NTBI to ineffective erythropoiesis

It can be seen from section 3.8 that In the case of thalassaemia syndromes, even though there are available iron binding sites on transferrin, NTBI is present in plasma. An explanation for this may be that as iron is released from macrophages after red cell catabolism in the bone marrow (ineffective erythropoiesis) or elsewhere (haemolysis) that the rate at which this occurs is too rapid for iron to bind to transferrin and other species of NTBI are formed. It is likely that the rate of oxidation of iron (II) to iron (III) is more rapid outside the bone marrow than within the bone marrow, where oxygen tensions are generally lower. Thus iron (III) binding to transferrin would be slower in the bone marrow, favouring NTBI formation when anaemia results more from ineffective erythropoiesis than haemolysis. Ineffective erythropoiesis and haemolysis both lead to anaemia and secondary expansion of intra and extramedullary erythropoiesis. However it is likely that ineffective erythropoiesis leads to a greater intramedullary expansion than with simple anaemia.

3.8.5.1 Relationship between NTBI and sTfR at low cumulative iron load in UCLH/HSC patients

Thus it is of interest to examine the relationship between sTfR and NTBI, especially in patients who have received relatively little iron loading from transfusion (where iron overload could mask a relationship between sTfR and NTBI). The most precise data on cumulative iron loading was available in UCL/HSC patients. It was decided to analyse the relationship between NTBI and sTfR in patients who had received relatively little iron loading (<1g) to see if there was a relationship between a marker of erythroid expansion (sTfR) and NTBI. 1g of iron is equivalent to 862 mls of pure red cells or 1440 mls of blood with an haematocrit of 0.6 (See TIF guidelines). The rate of transfusion in TM is 100-200mls /kg of pure red cells/y (TIF guidelines). Thus in young child with TM, 1g will take between 1 and 2 years to accumulate. It can be seen that there is a clear trend of increasing NTBI with increased erythroid expansion, consistent with the hypothesis that erythroid expansion overload the ability of transferrin to bind iron (III) at a sufficient rate to prevent NTBI formation.

Figure 3.8.5.a: Relationship of sTfR to NTBI in UCLH/HSC patients who had received less than 1g of iron from blood transfusion

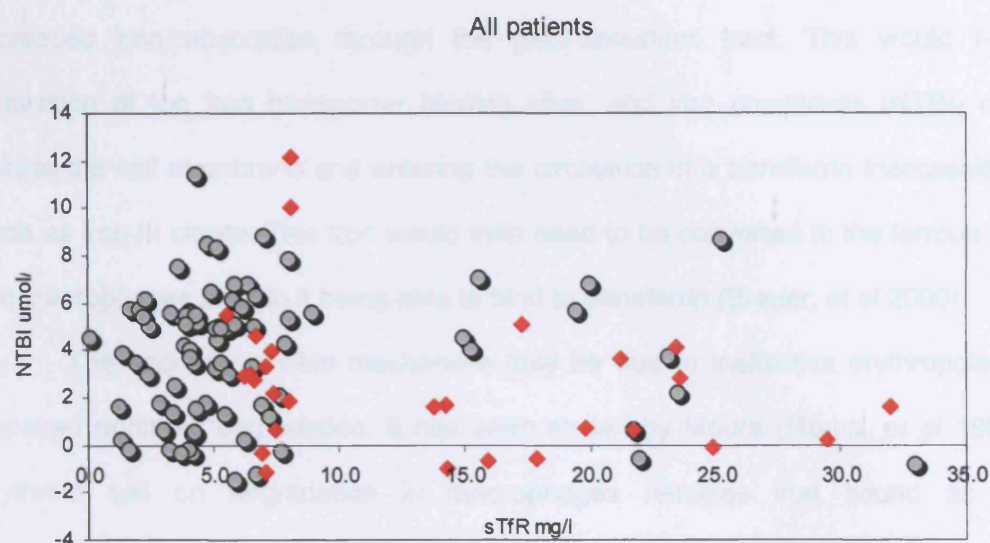


Legend: sTfR and NTBI in those patients from UCLH/HSC with cumulative iron less than 1g

3.8.5.2 Relationship between NTBI and sTfR for all patients groups

When NTBI and sTfR were analysed in all patients there was no correlation in both TI and TM patients (figure 3.8.5b)

Figure 3.8.5.b: sTfR and NTBI in TI and TM patients



Legend: NTBI and sTfR in all patients with simultaneous samples showing no correlation between these parameters $n=110$, (TM= 82; TI=28); $P=0.049$

TM black circles; TI red diamonds

3.8.6. Discussion: factors affecting NTBI

NTBI appears early in TM patients. In this study all the TM patients had detectable levels of NTBI regardless of the preceding transfusion history. In addition, NTBI was detectable in the TM patients' prior to start of transfusion and then fell to low levels prior to second transfusion at which point the anaemia was less profound. Thus ineffective erythropoiesis contributes to NTBI formation even in the absence of transfusional iron loading in thalassaemia syndromes.

Although there is no correlation between anaemia and NTBI, the presence of NTBI at this very early pre transfusion period at a time where the patient has profound anaemia is important. Two main mechanisms may account for this NTBI.

The first mechanism may be that the anaemia-associated hypoxia drives increased iron absorption through the gastrointestinal tract. This would result in saturation of the iron transporter binding sites, and iron complexes (NTBI) diffusing across the cell membrane and entering the circulation in a transferrin inaccessible form such as iron III citrate. This iron would then need to be converted to the ferrous form by the macrophages prior to it being able to bind to transferrin (Breuer, *et al* 2000).

The second possible mechanism may be due to ineffective erythropoiesis and impaired erythroid degradation. It has been shown by Moura (Moura, *et al* 1998) that erythroid cell on degradation in macrophages releases iron bound as ferritin, haemoglobin and a non protein bound iron complex described as Low molecular weight iron (LMW iron). Transferrin when exposed to this LMW iron bound it rapidly. In conditions of ineffective erythropoiesis where iron turnover is many times that of normal individuals it is conceivable that NTBI would be released from the degraded red cells and the rate of binding to transferrin may be too slow to keep up with the rate of efflux into the circulation. This is particularly likely in the hypoxic conditions of the bone marrow where oxidation of iron (II) to iron (III), and hence binding to transferrin is likely to be slower. In the TI patients NTBI is present at high levels similar to that seen with TM patients and the transferrin saturation tends to be lower (table 3.6.2.2). The finding of a trend of higher NTBI values in patients with higher sTfR, in patients with low transfusional iron loading is consistent with the idea that ineffective erythropoiesis contributes to NTBI formation. Probably both ineffective erythropoiesis and iron overload play an important role in the appearance of NTBI in both TI and TM patients and in the continued presence of NTBI in TI patients.

3.9 Conclusions

In thalassaemic children sTfR and EPO correlate well with the degree of the anaemia. There appears to be very little oxidative damage as measured by levels of MDA and protein carbonyls in these children who are on the whole well transfused and chelated.

There are some concerns regarding sample collection and handling in the Sri Lankan cohorts of patients. This is primarily because the sample needs to be collected and processed and then shipped to the UK for these markers to be measured. As discussed earlier all attempts were made to ensure that samples arrived in a frozen state with plenty of dry ice. In cases where this was unsuccessful and samples were not frozen, they were discarded. It is possible that the MDA levels are higher, although not abnormal in these patients because of sample activation. The assay used for measuring MDA measures total MDA as discussed earlier and is a relatively crude test. However because the levels are not raised compared to our normal control population we can probably assume that the free MDA levels will not be raised. Another concern is that there are no normal ranges for MDA or carbonyls in normal healthy children and our children results have been compared to those of normal healthy adults. The reason for the lack of age matched healthy control children are the difficulty in obtaining ethical approval for a blood test that is unnecessary in healthy children.

There is no relationship between markers of ineffective erythropoiesis and markers of oxidative damage. In addition neither of these factors have any correlation with the presence or absence of NTBI. The relationship between markers of oxidative damage and iron burden in children is at best tenuous. Markers of oxidative damage

have been found to be raised in adult thalassaemic patients. In our study in young children who are on regular chelation regimes, markers of oxidative damage were not raised. Chelation therapy results in a shorter duration of exposure to toxic forms of iron as NTBI is removed from the circulation, in adults however there has been a longer duration of exposure to toxic iron species as they received less chelation therapy in childhood.

The presence of NTBI early in thalassaemia patients is an important observation in this study and has not been described previously. It is likely, based on the analysis of data available, that ineffective erythropoiesis is an important contributing factor to NTBI formation in patients who have accumulated only small quantities of excess storage iron. It will be important to continue with the longitudinal follow-up that is part of this study to see if oxidative damage increases with age and prolonged exposure or if a patient becomes non adherent to therapy and develops significant iron burden.

Chapter Four

Relationship of hepatic iron and NTBI to myocardial Iron and LV function; contrasts between sickle and thalassaemia disorders

4.1 Introduction

The most serious and frequently fatal consequence of transfusional siderosis in thalassaemia major is cardiac failure or dysrhythmia due to iron deposition in the myocardium. This is well recognised and documented in the literature for transfusion dependant thalassaemia (Aldouri, *et al* 1990, Brittenham, *et al* 1994, Davis and Porter 2000, de Montalembert, *et al* 1989, Fitchett, *et al* 1980, Modell, *et al* 2000). However, while transfused patients with SCA are known to have increased iron burdens, as measured by liver biopsy, cardiac complications secondary to iron overload are rarely described (Batra, *et al* 2002).

Unlike patients with TI who may have similar steady state Hb values, in the absence of blood transfusions, patients with SCA do not become iron overloaded (Harmatz, *et al* 2002, Vichinsky, *et al* 2005) However they may require intermittent or regular blood transfusions as a part of the management of their disease as discussed in chapter one. Indications for transfusion may include rapid onset severe anaemia, chest syndromes, sickle stroke syndromes, priapism, and in preparation for surgery (Danielson 2002, Reed and Vichinsky 1999, Riddington and Wang 2002, Sandoval, *et al* 2002,

Simmons, *et al* 1988, Vichinsky 2002). Transfusion regimens in such cases may either be in the form of an exchange transfusion or top up transfusion.

Recognised complications of transfusion regimens in SCA are similar to those seen in the general population who may receive blood and are antibody formation against red cell blood groups due to the variation between the donor and recipient red cell phenotypes, transfusion reactions, transfusion transmitted infections and iron overload (Danielson 2002, Ohene-Frempong 2001, Olujohungbe, *et al* 2001, Sandoval, *et al* 2002). However, there is little published data on the relationship between iron overload and its complications, particularly to the myocardium (Wood, *et al* 2004). This is important to understand because blood transfusion is being used increasingly as a therapeutic modality, particularly in patients at risk from stroke (Adams, *et al* 1998a).

There is remarkably little data available on the effects of blood transfusion on iron distribution in SCA. Kupke-Faget (Kulpe-Faget, *et al* 2003) reported iron loading in the myocardium at *post mortem* in patients with SCA and found that three of seven patients with severe hepatocellular iron loading had myocardial iron deposition and one other patient had moderate or lesser levels of siderosis. In three out of these four patients there was also evidence of myocardial fibrosis and cell degeneration compared to five of twenty seven patients without myocardial iron loading. In an MRI study using a T2* technique (see below) Wood reported normal cardiac T2* in all of 17 patients receiving long-term transfusions for SCA (Wood, *et al* 2004). However, on echocardiography Batra did find some evidence of impaired left ventricular diastolic function in chronically transfused patients with SCA and suggested that this was one of the early signs of iron loading in the myocardium (Batra, *et al* 2002).

It has been proposed that iron distribution may differ between patients with SCA and thalassaemia and that this may be due in part to the inflammatory response seen in SCA (Finch, *et al* 1982, Harmatz, *et al* 2002). There are significant changes in

the cytokine profiles in patients with SCA both during the acute vaso-occlusive episode and in the steady state when the patient is clinically well. It was shown by Taylor et al (Taylor, *et al* 1997) that type II cytokines such as IL4, 6, and 10 were raised in SCA during the steady state.

It is known that IL6 is a potent inducer of hepcidin (Nemeth, *et al* 2004). Hepcidin synthesis is up-regulated in the anaemia of chronic disease by IL6 and other cytokines (Ganz 2006). Furthermore, hepcidin down regulates iron absorption from food by decreasing iron release from enterocytes and release of iron from macrophages (Nicolas, *et al* 2001). It is therefore possible that the existence of a chronic inflammatory state in SCA, leads to sequestering of iron in the RE cells and consequently a reduced transferrin saturation and NTBI, even in the presence of transfusional iron overload. This would result in a decreased probability of iron uptake into tissues that possess uptake mechanisms for NTBI, such as the myocardium and endocrine tissues.

TI patients can also develop significant iron overload when minimally transfused or even when untransfused, due to increased iron absorption from the gastrointestinal tract (Bannerman, *et al* 1967, Pippard, *et al* 1979). The signal for this increased iron absorption is thought to be related to the degree of ineffective erythropoiesis as discussed in chapter 3 and it may be that the severity and type of anaemia override iron overload mediated down regulation of iron absorption.

Iron burden has historically been assessed by liver biopsy as liver iron concentration (LIC) accurately predicts total body iron stores (Angelucci, *et al* 2000). Studies on the distribution of iron in different tissues have generally been on post mortem sampling. Myocardial biopsies in patients with iron overload have not been performed routinely because of the practical difficulties in obtaining tissue samples. More recently however, with improvements in image acquisition, MRI has been used to assess liver and cardiac iron in patients with haemoglobinopathies in a non-invasive manner.

This has allowed clinicians to gain an insight into which patients may be at risk of cardiac complications due to iron deposition (Anderson, *et al* 2001, Galia, *et al* 2003, Jensen, *et al* 2001, Papakonstantinou, *et al* 1995) .

A key objective in this chapter has been to apply the newly available non-invasive techniques of liver and heart iron estimation, using the T2* technique, to explore whether the relationship between transfusional iron loading and tissue iron accumulation is the same for sickle cell and thalassaemia disorders.

4.2 Techniques for estimating myocardial iron

4.2.1 Endomyocardial biopsy

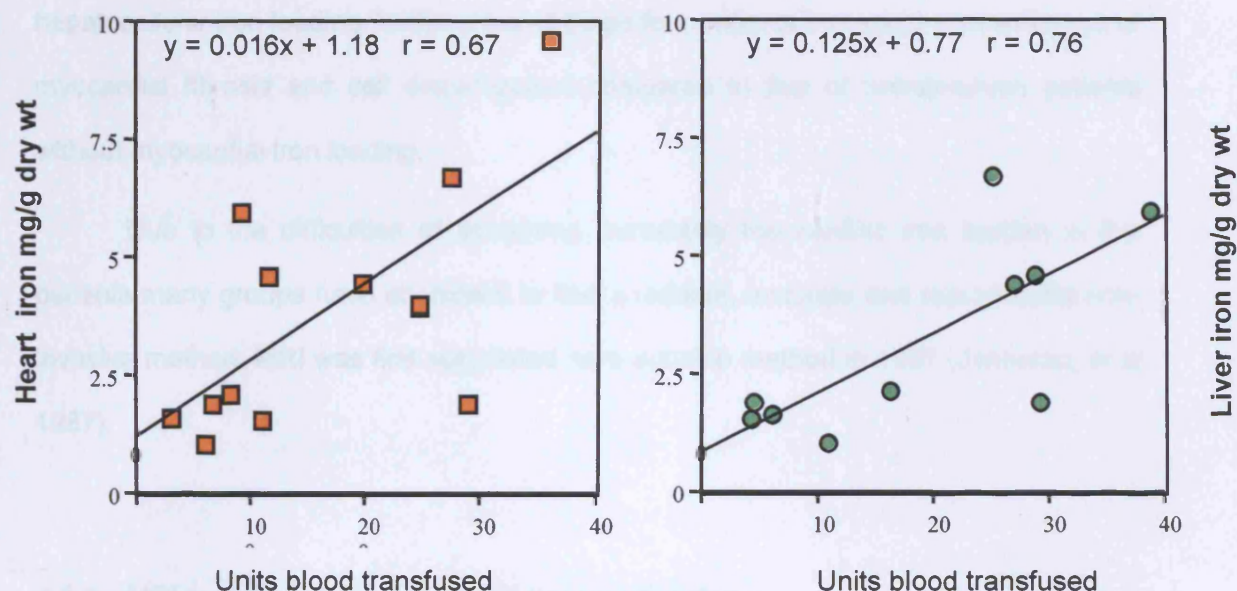
Cardiac biopsies are performed using standard right heart catheterization techniques and the biopsy samples are predominantly of the right interventricular septum. It is an invasive but relatively safe technique for assessing cardiac pathology and has been used extensively over many years (Fowles and Mason 1982, Frustaci, *et al* 2002). The usefulness of endomyocardial biopsy in looking at cardiac iron burden has been assessed in a number of studies. An Italian study by Lombardo (Lombardo, *et al* 1995) looking at 15 patients with thalassaemia found some correlation between myocardial iron grade and ferritin and suggested this was a useful technique in assessing transfusional haemosiderosis, It did not however look at the distribution of iron throughout the myocardium. An earlier study on 4 severely iron overloaded TM patients by Fitchett (Fitchett, *et al* 1980) showed a raised iron concentration in only one patient on endomyocardial biopsy and a patient with cardiac failure secondary to iron overload had a normal iron concentration. They concluded that endomyocardial biopsy was an insensitive method because of the variability of iron distribution and sampling.

Endomyocardial biopsies have been performed on patients with sickle cell anaemia (Tap, *et al* 2001) but transfusional haemosiderosis was not assessed.

4.2.2 Post mortem studies

Post mortem studies have provided the most information on the distribution of iron in the myocardium. Buja and Roberts (Buja and Roberts 1971) in their classical paper, described post mortem review of deaths in patients with chronic anaemia who require transfusions and patients with haemochromatosis. They found variability of iron distribution throughout the heart, with the iron loading in the ventricular tissue first followed by the atrial tissue as iron burden increased. The ventricular iron was primarily in the epicardial region, of intermediate concentration in the subendocardial region and least in the middle third of the ventricular walls. Iron deposits were found in the cardiac myocytes and the interstitial tissue as well as in the conducting bundles. The predominant form of iron in the myocardium was haemosiderin. The study of Buja and Roberts, in the pre-chelation era, showed that in the adult population of 131 patients mainly with acquired bone marrow failure disorders such as leukaemia, that increased haemosiderin was seen in 30% of patients who had received 50-75 units of blood, 60% of patients who had received 65-200 units, and 100% of patients who had received in excess of 200 units. In some of the patients, quantitation of liver and myocardial iron was performed. Figure 4.2.2 show the relationship of myocardial iron concentration to LIC and to units of blood transfused obtained from data in Buja and Roberts, 1971.

Figure 4.2.2a: cardiac and LIC correlations from pre-chelation era



Legend: The relationship between heart iron (mg/g dry wt) and liver iron quantification at post mortem with units of blood transfused was ($r=0.67$) or ($r=0.76$) respectively as shown for patients with mainly acquired anaemia associated with a variety of 'leukaemia's' (Buja and Roberts, 1971).

Tashiro et al showed a reduction in the siderotic granules as well as an ultrastructural improvement of myocyte vacuolation and disarray after 3 months of intensive chelation with desferrioxamine (Tashiro, *et al* 1990).

The majority of biopsies studied in SCA have been post mortem myocardial biopsies in cases of sudden death and have shown myocardial infarction and myocardial necrosis secondary to occlusion of the cardiac microvasculature by sickled red cells (Mansi and Rosner 2002, Martin, *et al* 1996). Kupke-Faget (Kulpe-Faget, *et al* 2003) studied iron loading in the myocardium in patients with SCA and found that out of a total

of 31 patients seven had severe hepatocellular iron loading and 24 had moderate or mild hepatic iron loading. Three of the seven had myocardial iron loading and only one patient had myocardial loading out of the twenty four patients with mild to moderate hepatocellular iron loading. In three out of these four patients there was also evidence of myocardial fibrosis and cell degeneration compared to five of twenty-seven patients without myocardial iron loading.

Due to the difficulties of assessing accurately the cardiac iron burden in live patients many groups have attempted to find a reliable, accurate and reproducible non-invasive method. MRI was first suggested as a suitable method in 1987 (Johnston, *et al* 1987).

4.2.3 MRI techniques for assessing myocardial iron

4.2.3.1 Principle

The use of MRI to estimate tissue iron relies on the paramagnetic properties of iron ferritin and hemosiderin iron. MRI measures either T1 (longitudinal) or T2 (transverse) weighted relaxation times of water molecules (H atoms). Iron overload in tissues results in shortening in the longitudinal axis (T1) and transverse (T2) tissue relaxation times on which magnetic resonance imaging is based and leads to a reduction in signal intensity. The decrease in the intensity of spin echo images with iron overload results from shortening of the T2 relaxation times (Stark 1991). This shortening is mainly due mainly to the paramagnetic properties of ferritin iron.

It is likely that different forms of iron have different effects on T1 and T2 relaxation times. While inorganic iron (III) shortens both T1 and T2, this effect is much more pronounced

when the same amount of iron is present as ferritin (Brown, *et al* 1985) as ferritin is water soluble. By contrast, hemosiderin, a degradation product of ferritin, is water-insoluble with a larger cluster which probably explain lack of significant T1-shortening effect of this molecule on T1-weighted images (Vymazal, *et al* 2000). Ferrioxamine, which is one of the products of ferric iron binding to DFO, is also paramagnetic, and is used as a contrast media using predominantly T1 weighed sequences (Duewell, *et al* 1991, Muetterties, *et al* 1991, Tian, *et al* 1997, Worah, *et al* 1988).

4.2.3.2 Image acquisition

MRI has been used for a number of years to assess cardiac function and cardiac iron burden using a number of different parameters and techniques (Anderson, *et al* 2001, Jensen, *et al* 2001, Liu, *et al* 1996, Wood, *et al* 2004). As MRI methodology has become more refined, sensitivity has increased and good correlations are now seen with liver MRI assessments and liver biopsy iron quantification. Two different types of echo sequences (Spin and Gradient) are used for image acquisition and both have advantages and disadvantages (see below).

Spin Echo

This is the most widely used sequence and uses both 90 and 180 flip echo sequences. The use of both 90 and 180 ° flip echo results in a reduction in field inhomogeneities and return a good echo signal but the acquisition times are longer and 3D images cannot be acquired.

Gradient Echo

This uses the simplest sequence to obtain an echo signal (alpha flip-gradient recalled echo). It is used mainly to acquire T2* decayed based images. The main

advantage to this technique is that image acquisition is faster and more images can be acquired. Artefacts due to wall motion are reduced and 3D images can be taken. The disadvantages are that it can be difficult to generate good T2 weighting and magnetic field inhomogeneities can cause signal loss.

4.2.3.3 *Signal analysis*

The echo signals can be analyzed in a variety of ways such as the T2 signal alone, as a ratio with a control tissue such as muscle, or using mathematical formulae to calculate the T2*. Hence there are a number of combinations that can be used for Iron quantification based on these variables and a universal method that is reliable and reproducible has yet to be agreed for measuring myocardial iron.

Cardiac T2 and SIR

The first method to be used for assessment of cardiac iron was the T2 (Perrimond, *et al* 1991) in which T2 images were acquired using spin echo for both the liver and the myocardium. This showed that measuring T2 of the myocardium was feasible however the technique needed further refinement due to problems with wall motion artefacts and poor signal to noise ratios at longer echo times.

As methodology has become more refined wall motion artefact and gating of image acquisition with the cardiac cycle so that images are acquired at the same time in each cycle has helped to limit some of the initial problems with measuring cardiac relaxation signals and T2 measurements or T2 as a ratio with muscle (SIR). Both Jensen and Mavrogeni (Jensen, *et al* 2001, Mavrogeni, *et al* 1998) have shown correlation of liver T2 with the cardiac T2 or myocardial/ muscle SIR. In addition Jensen showed a correlation between number of units of blood given and myocardial iron

content as calculated by using a modified liver calibration curve in patients who were transfused but not chelated.

*Cardiac T2**

T2* measured using gradient echo was described by Anderson et al (Anderson, *et al* 2001). Anderson chose gradient echo because of the greater sensitivity to iron and the shorter acquisition times resulting in less motion artefact and is thought to be more reproducible than SIR. T2* is related to T2 by the summation of the tissue relaxation time (T2) and the magnetic inhomogeneity known as T2 prime (T2') by the formula:

$$1/T2^* = 1/T2 + 1/T2'$$

In this study a strong correlation was found for liver iron quantification and liver T2* value (R=0.93 in non fibrotic liver samples). The study could not use myocardial iron validation to quantify myocardial iron due to the problems associated with endomyocardial biopsy as discussed in section 4.2.1 but found a significant correlation with other parameters of myocardial function specifically ejection fraction and end systolic volume supporting the fact that cardiac T2* was of prognostic importance.

Magnetic transfer ratio (MTR)

Papanikolaou et al (Papanikolaou, *et al* 2000) used this method to assess cardiac iron burden. This methodology uses gradient echo sequences for image acquisition. MTR measures the transfer of magnetization between the tissue being imaged and the water molecules in that tissue, a low transfer reflects reduced magnetization and hence damage. Papanikolaou found that patients with TM had significantly reduced MTR values compared to normal healthy controls.

4.2.4 Discussion

Recently tissue validation has been undertaken in humans with Mavrogeni *et al* validating T2 with endomyocardial biopsy (Mavrogeni, *et al* 2005). These patients presented with NYHA grade II or III heart failure and the validation showed a good relationship between T2 and cardiac iron. There has only been a single case report of endomyocardial biopsy and cardiac T2* (Westwood, *et al* 2005) although studies are underway currently to validate T2* with endomyocardial biopsies. There is also some recent animal data by Wood *et al* in iron-overloaded gerbils using T1, T2 and T2* confirming a linear increase in cardiac iron burden with increasing iron load (Wood, *et al* 2005).

The tissue validation of myocardial iron is becoming clearer due to the above studies but concerns remain with the regards the distribution of iron as noted by a recent MRI study using T1, T2, T2* and SIR (Macarini, *et al* 2005). In addition, the effect of ferrioxamine that is present in tissues following chelation with desferrioxamine and its effect on the signal intensity is as yet unclear.

Advances in MRI methodology have therefore lead to significant advances in our understanding of iron distribution. There is however considerable concern over certain observations noted in some of the studies. The main concern is the lacks of correlation between the traditional parameters of iron overload such as the liver iron burden and the serum ferritin seen in Anderson's study. The lack of correlation of myocardial T2* with liver iron in a cohort of well-chelated patients may be due to a number of reasons. Most importantly is the effect of chelation history on iron burden. In Jensen's study the

patients had been followed from the start of iron chelation therapy and the longitudinal response assessed. In Anderson's study a cross-sectional assessment of the parameters was undertaken with no review of prior chelation history.

It is known that serum ferritin reflects hepatic iron burden in iron-loaded individuals (Borgna-Pignatti and Castriota-Scanderbeg 1991) but it is also well recognized that ferritin is an acute phase protein and not ideal as a monitoring tool in patients with chronic conditions. It is most probable that in those patients who had myocardial iron loading previous chelation had been poor allowing iron to accumulate in the myocardium (Gabutti and Piga 1996). Brittenham (Brittenham, *et al* 1994) had previously shown that patients with liver iron burdens greater than 15 mg/g dry weight were at risk of cardiac failure and those patients with a history of poor chelation may iron load into the myocardium. These patients would have had high ferritin levels or falling ejection fractions initially and this would have prompted intensive chelation to be instituted and hepatic siderosis would have improved more rapidly because desferrioxamine is an efficient hepatic chelator, the myocardial iron is removed more slowly (Porter and Davis 2002). A significant number of the patients in Anderson's study were those undergoing intensive chelation as described by Davis and Porter (Davis and Porter 2000). In order to address the concerns raised by the cross-sectional study it was decided to assess the longitudinal data from the MRI assessments as outlined in section 4.3.

4.3 Aims and Rationale of study

The broad aim of this chapter was to investigate whether transfusional iron loading in SCA has different consequences from iron loading in TM or TI. The recent development of MRI techniques to estimate heart and liver iron, as well as left ventricular function, allows for the first time an opportunity to examine how total body iron, as estimated from liver iron concentration (LIC), impacts on heart iron as estimated from myocardial T2* and on heart function as measured by left ventricular ejection fraction (LVEF) .

Cross sectional analysis of the relationship between liver iron concentration (LIC) and myocardial T2* has emphasised the lack of correlation in multi transfused, multi-chelated patients (Anderson, *et al* 2001). However it is clear from further studies by the same group that chelation therapy with desferrioxamine normalised liver iron as estimated by T2* much more rapidly than heart T2* (Anderson, *et al* 2004) . Furthermore studies using MRI in multi-transfused patients naive to chelation therapy show a clear relationship between liver iron and myocardial iron using SIR (Jensen, *et al* 2003). In this chapter, an important part of the analysis has been to examine how these variables change with time in relation to total iron loading and in relation to chelation therapy.

The only MRI centre quantifying cardiac iron loading in the UK uses the cardiac T2* technique and this has been used to monitor patients at UCLH since 1999, allowing an analysis of factors which may influence the accumulation or removal of iron from the heart in both thalassaemia and sickle patients treated at UCLH.

There were 3 specific aims in this study.

Specific Aim 1: The first aim was to examine the relationship between heart iron, liver iron and heart function in thalassaemia patients by cross sectional analysis.

Specific aim 2: The second aim was to establish factors that affect changes of cardiac T2* with time in thalassaemia patients. Factors which have been examined are:

- a) The effect of chelation therapy.
- b) The influence of body iron burden (LIC) on improvement or worsening of cardiac T2* with time, irrespective of the dose or duration of chelation therapy.

Specific aim 3: The third aim was to see if iron distribution between heart and liver in multi-transfused SCA patients was different to that seen in TM with similar levels of iron loading and if so to identify reasons why this might arise. In principle differences in iron distribution to the heart could relate to differences in duration of transfusional overload, effects of chelation history or to fundamental differences in iron metabolism between sickle and thalassaemia syndromes. One aspect of the latter is whether NTBI is related to iron overload differently in SCA compared to thalassaemia syndromes. To address this, we compared cardiac T2* in patients matched for liver iron to see if the cardiac T2* values were different in the sickle and TM patients and a history of iron associated complications noted. NTBI and transferrin saturations were measured in the matched

pairs and compared. Patients with TI were assessed separately because it is difficult to accurately assess the rate of iron loading due to variable gastrointestinal iron absorption.

4.4 Patients

4.4.1 Patients for specific aim 1

Between July 1999 and November 2002, a total of 98 patients with TM (n=75) or TI (n = 23), followed at UCLH, had at least 1 assessment of cardiac and liver T2* together with LVEF estimation by MRI at the Royal Brompton Hospital CMR unit. The population of patients was 51 females and 47 males and the mean age was 27.1 years (range 6.7- 53.7 yrs). For the purposes of this analysis, TM patients were those who had a severe transfusion dependant anaemia requiring blood transfusions at a 2 to 4 weekly interval. TI patients were defined as those with homozygous beta thalassaemia or compound heterozygote (Hb E β thalassaemia) who were transfused <8 times per year or were transfusion independent.

All transfusion dependant patients (TM) were on regular chelation support adjusted according to the ferritin and body weight. Only 3 patients from the 98 received deferiprone, 2 as part of a combination regime with desferrioxamine and one as monotherapy. The remainder of the patients were chelated with desferrioxamine.

4.4.2 Patients for specific aim 2

Retrospective analysis over a 3 year period was undertaken on those patients with a low or borderline cardiac T2* on first measurement or on those who subsequently

developed a low cardiac T2*. 42 patients with TM had a low cardiac T2* (<20ms) on the preliminary scan and 1 developed a low cardiac T2* on a subsequent scan. Of these, 13 patients had had 4 or more CMR assessments at 6 monthly to yearly intervals (12 TM and 1 TI patient). The rate of improvement of cardiac T2* was calculated for these patients who were on intensive chelation regimes at severe, moderate and mild levels of cardiac iron loading to see if the rate of improvement was altered by levels of loading and if there was a relationship between this and liver iron burden. One of the 13 patients agreed to undergo sequential CMR assessment on DFO and after stopping DFO to see if cardiac T2* or liver iron was significantly affected by delayed elimination of ferrioxamine.

4.4.3 Patients for specific aim 3

26 Patients with SCA were studied and included either Hb SS (n=20), S β thalassaemia (n=2) or HbSC (n=2) and HbSD (n=2). These patients, who were receiving top up or exchange transfusions or who had been transfused in the past or had a history of cardiac arrhythmia, underwent CMR for assessment of iron overload. Transfusion history, history of cardiac complications and chest syndrome were collected from a review of hospital blood bank records, patient notes and interviews with the patients. Iron burden calculation took into account loss of iron by venesection as part of exchange transfusion. Most patients were on a 3 units-in 4 units-out regimen for exchange and most patients had had a combination of exchange and top up transfusions. These patients were matched for age and liver iron with 26 TM patients who were receiving blood transfusions at two to four weekly intervals. All of these patients were on regular iron chelation therapy with DFO. Only one of the TM patients was on combination therapy with Deferiprone. Histories of cardiac abnormalities were collected from patient's

notes and interviews. Cumulative iron load was calculated for the matched pairs from the units of blood received but the use of iron chelation therapy could not be taken into account in the calculation of cumulative iron load.

Thirteen TI patients had had CMR assessment. Transfusion history was collected in a similar manner as above along with history of cardiac and endocrine complications. These patients were analyzed separately and were not part of the full matched pair study because iron loading can occur independent of transfusion.

4.5 Methods

4.5.1 Cardiac T2*

Patients underwent CMR using a gradient spin echo technique as described by Anderson *et al* for the measurement of myocardial T2*. A single short axis midventricular slice was acquired using a gradient echo sequence on a Siemens Sonata 1.5T MRI scanner at nine separate Echo times (TE 5.6 –18.0ms). The signal intensity of this region was plotted against the echo time used for each image. The resulting points form an assumed exponential decay curve, as the image signal decreases with increasing echo time. An exponential function was fitted to the data, with the equation being in the form:

$$y=Ke^{-TE/T2^*}$$

Where K represents a constant, TE represents the echo time and y represents the image signal intensity.

For diagnosis of increased cardiac iron burden a T2* of 20ms or greater suggests no iron deposition in the myocardium and less than 20ms suggests that iron is present, levels less than 8 ms imply severe myocardial iron loading.

4.5.2 Left Ventricular Ejection Fraction

Manual tracing of epicardial and endocardial borders of contiguous short-axis slices at end-diastole (first cine phase of the R-wave-triggered acquisition) and end-systole allowed calculation of LV mass, LV end-diastolic volume, and LV end-systolic volume, from which LV stroke volume and ejection fraction were derived.

4.5.3 Liver T2*

For the measurement of liver T2* a single transaxial 10mm slice through the centre of the liver was acquired using a gradient echo sequence at eight different echo times (2.2 - 20.1ms). The echo times were used to calculate the T2* using the same formula as in section 4.5.1 and Liver iron in mg/g dry weight was calculated by the formula:

$$\text{Liver iron} = \text{EXP}(2.66409 - (1.06611 * \text{Log}(\text{liver T2* ms})))$$

4.5.4 Statistical analysis

The mean, geometric mean standard deviation and standard error of the mean were calculated for each cohort of patients. Student T test were used to calculate significance. For paired analyses the paired T test was used.

4.5.4.1 *Calculation of cumulative iron burden*

Some assumptions were made in the calculation of cumulative iron. During 1980 to 1986 blood in the UK was gradually changed from whole blood donations to packed cells. For the purpose of the analyses it was assumed at all blood was administered in the form of packed cells after 1/1/86 and all blood prior to this was whole blood. The haematocrit of packed cell units was assumed to be 0.60 and that of whole blood 0.40. The mls of blood in a whole blood unit was assumed to be 450 ml and that of a packed cell unit 350ml.

Thalassaemia major patients:

The total units of blood transfused was collected from the patient records system as far back as the pathology system allowed (1980) with those units transfused prior to 1/1/86 used to calculate cumulative iron separately. This was done for the majority of patients. In those patients who had not been transfused at UCLH or where records did not go all the way back to initial transfusions assumptions were made in calculation of units of blood transfused based on average blood usage for a child of set ages as follows. Assuming that 1 unit of blood was administered monthly from age of first transfusion up to the age of 10 yr (this assumption would balance out the smaller volumes administered in early childhood with the larger volumes administered in late childhood and was born out by the data collected for the children in Chapter 3). Blood usage from the age of 10yrs to 16 years was assumed to be 2 units every 4 weeks if data was not available and dependent on information provided by patients on direct questioning. Cumulative iron load was calculated according to the formula as described by Porter (BJH 2001)

$$\text{Iron content of transfusion (mg)} = \text{volume (ml)} \times \text{haematocrit} \times 1.16$$

The loss of iron via chelation was not included in the assessment of cumulative iron load because chelation therapy adherence was variable and clear histories going back through the life of the patients were not available. In addition the older thalassaemia patients had not started chelation until the mid 1970's. At that time DFO was administered as IM bolus injections and the method of administration was switched to subcutaneous treatment in the late 1970s and early 1980's.

Sickle cell anaemia

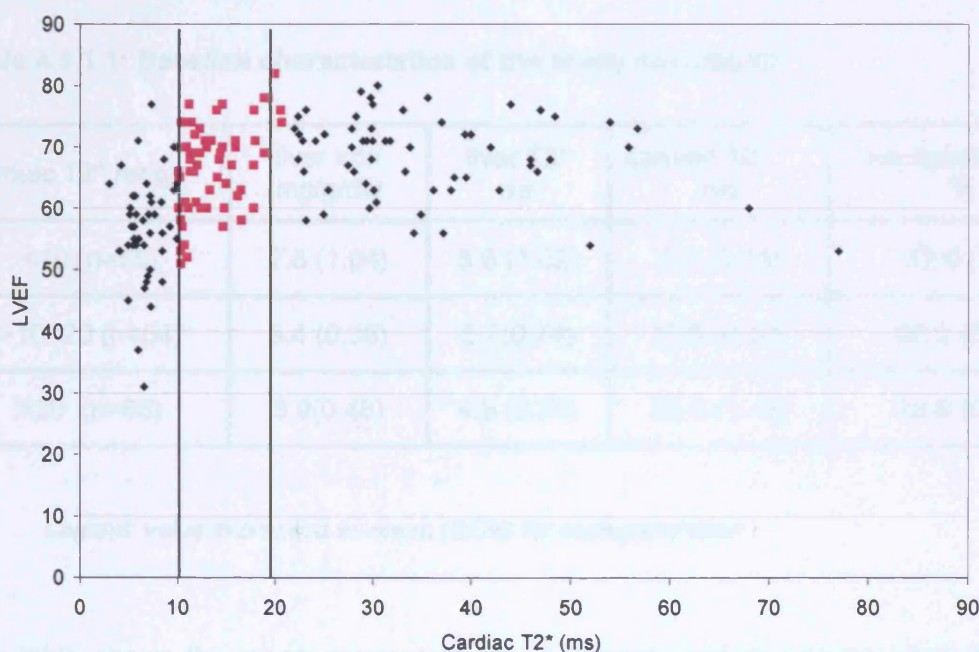
The same principles were used to calculate Iron load from blood transfusion and transfusion data collected in a similar manner. In addition as SCA patients often present to other hospitals a more detailed questioning was undertaken of past transfusion history including units of blood administered and if these were as part of exchange transfusions or top up transfusions. There are clear limitations to this method of data collection as patients were asked to provide information as far back as they could remember. It has to be accepted that some transfusions may have been missed. Most patients were on a 3 units-in 4 units-out regimen for exchange and majority of the patients had had a combination of exchange and top up transfusions. It has been standard practice to undertake pre and post transfusion HbS% assessments and it was fairly easy to work to how many units had been used for exchange transfusion and how many had been used for a top up transfusion. Calculation of cumulative iron load was carried out using the same formula as for the TM patients but took into account loss of iron by venesection as part of exchange transfusion as part of a separated calculation. To calculate the iron lost by venesection in exchange transfusions the haematocrit of the unit of blood removed was the mean of the pre-transfusion full blood counts. The cumulative iron load was

calculated by subtracting the iron loss from venesection once the separated calculations were done to give the actual transfusional iron load.

4.6 Factors affecting relationship between liver and myocardial T2* and heart function in Thalassaemia

4.6.1 Cross sectional analysis of the relationships between liver and heart T2* and LVEF in Thalassaemia major and intermedia.

Figure 4.6.1.a Relationship between Ejection Fraction and cardiac T2*



Legend: Cardiac T2 in 98 patients who underwent 170 assessments for myocardial iron assessment from UCLH, using the method described by Anderson et al. Pink squares: patients with cardiac T2* between 10 and 20 ms*

4.6.1.1 Proportion of patients myocardial T2* <20ms and >20ms

It can be seen from figure 4.6.1a that of 170 assessments that had all results for liver iron, cardiac T2*, and ejection fraction available for analysis, 61.1% have T2* values <20ms and 29% have T2* values <10ms. A cut off of 20ms was identified by Anderson et al (Anderson, et al 2001) as a value below which there was an increased chance of a decreased ejection fraction. Table 4.6.1.1 shows the mean values for the liver iron mg/g/dw and cardiac T2* for the patients.

Table 4.6.1.1: Baseline characteristics of the study population

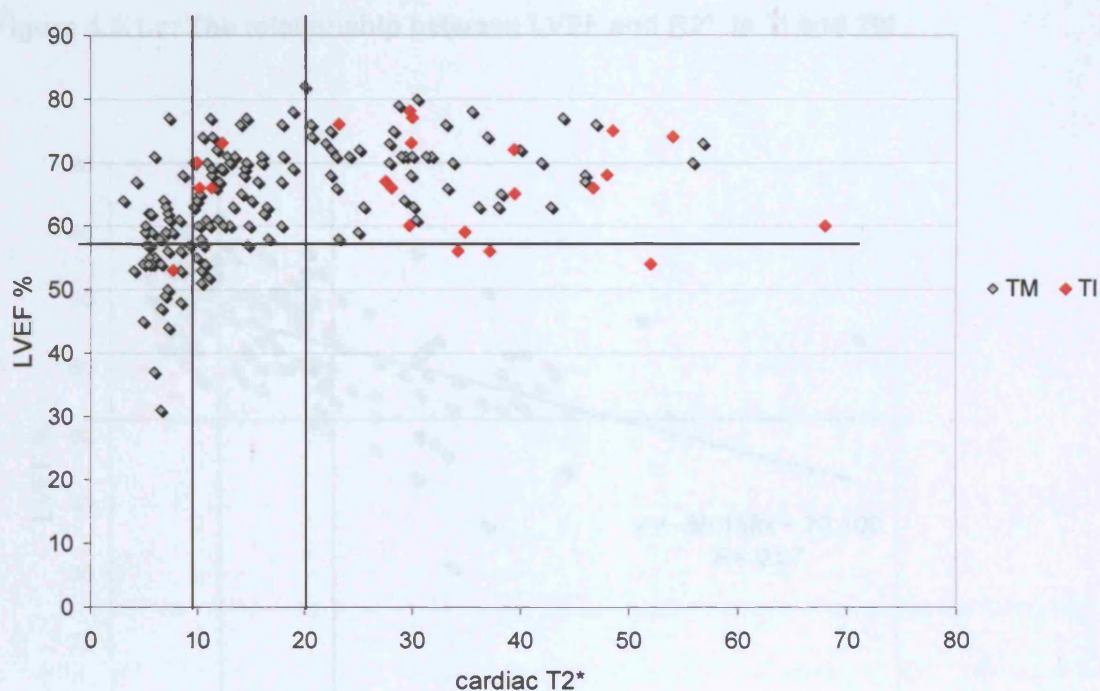
cardiac T2* range	liver iron mg/g/dw	liver T2* ms	cardiac T2* ms	ejection fraction %
<10 (n=50)	7.3 (1.04)	5.6 (1.02)	6.8 (0.23)	57.0 (1.2)
>10<20 (n=54)	5.4 (0.38)	5.7(0.74)	13.5 (0.38)	66.2 (0.96)
>20 (n=66)	5.9(0.48)	4.8 (0.74)	35.2 (1.49)	68.8 (0.81)

Legend: value expressed as mean (SEM) for each parameter.

This table shows the mean parameters for all patients and as can be seen the low cardiac T2* are associated with a low ejection fraction.

4.6.1.2 Relationship of LVEF to cardiac T2* and 1/T2* in TI and TM

Figure 4.6.1.b: The relationship between LVEF and cardiac T2* in TI and TM



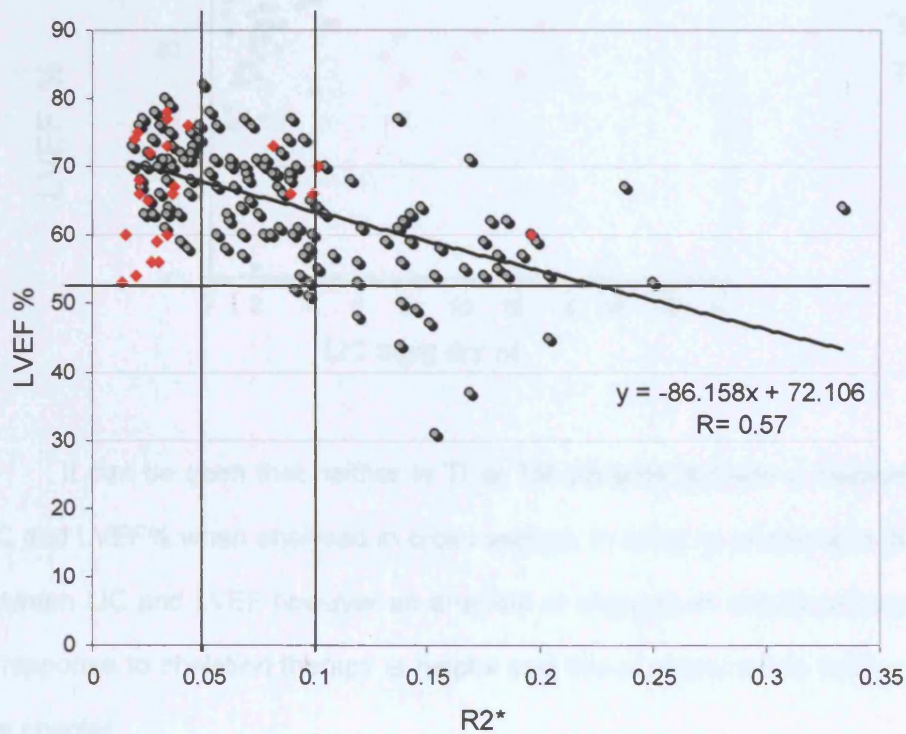
Legend: same figure as in 4.6.1a but showing TI and TM values separately All TI and TM patients with cardiac T2* and LVEF
 TI; red diamonds TM black squares

It can be seen that the relationship of myocardial T2* to LVEF% differs for TI and TM. In TM patients LVEF <56% is seen mainly in patients with T2* values ≤10ms. In TI patients 5 measurements have LVEF ≤ 60% despite T2* values >20ms (4

measurements from a patient with pulmonary hypertension and 1 from E β thalassaemia patient who has not been transfused). Furthermore two TI patients have T2* values < 10ms.

Because tissue iron is related linearly to $1/T2^*$ rather than $T2^*$ (Anderson, *et al* 2001) the same data as above was plotted for $1/T2^*$ or $R2^*$. It can be seen that LVEF of less than 53% are confined to patients with $R2^*$ values >0.1 (<10ms)) and that all these patients have TM rather than TI.

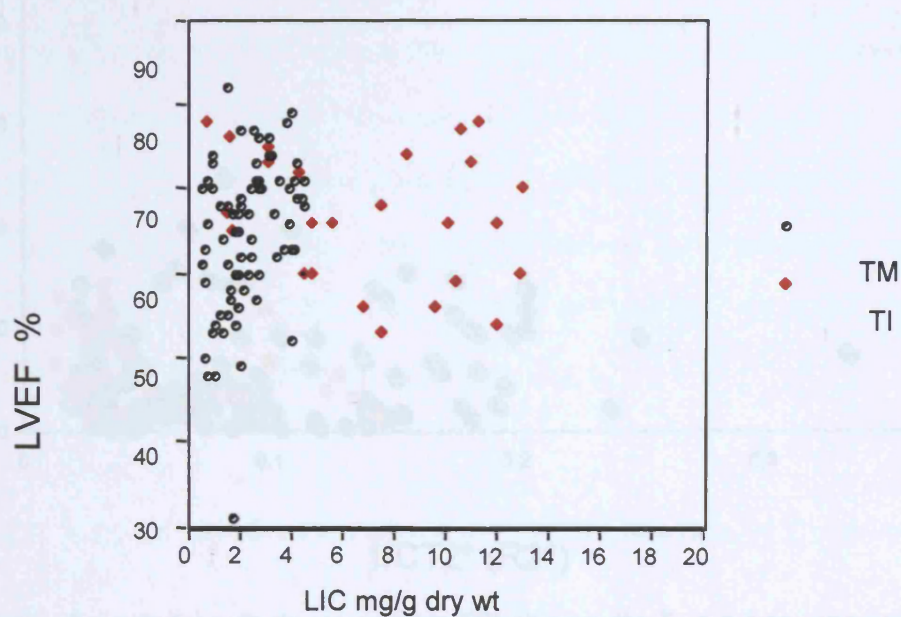
Figure 4.6.1.c: The relationship between LVEF and $R2^*$ in TI and TM



Legend: all results for patients converted to $R2^$ showing the TI in red squares and TM as black diamonds. This shows a good correlation with the LVEF $R=0.57$; $P<0.0001$; $n=163$*

4.6.1.3 Relationship of LIC to LVEF in TI and TM

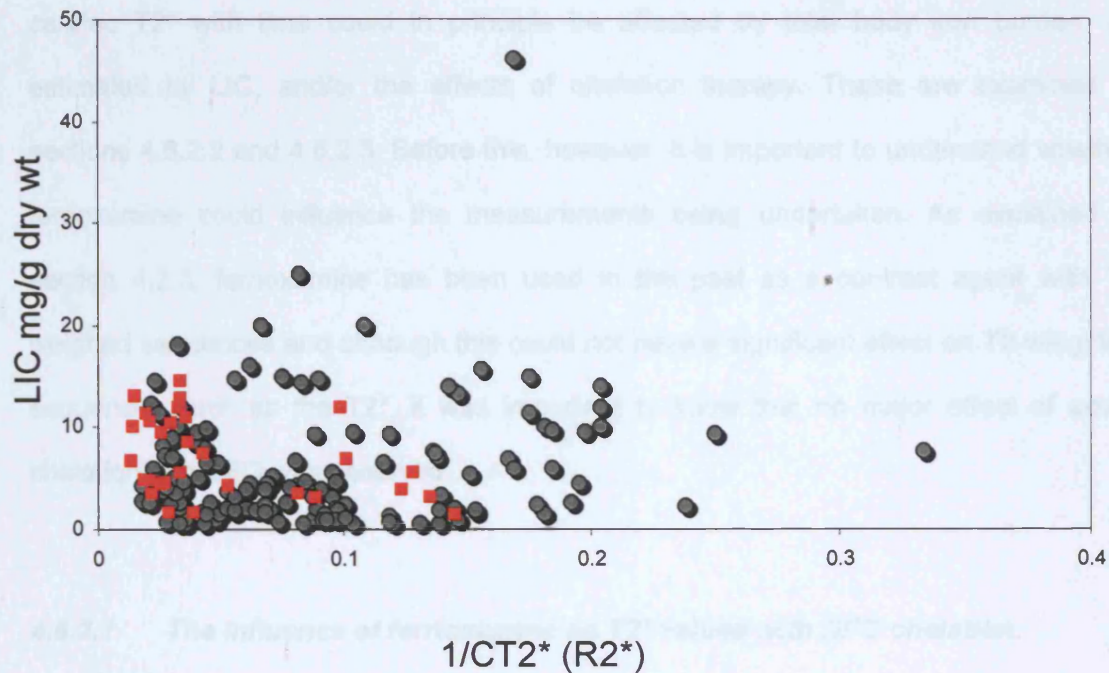
Figure 4.6.1.d: The relationship between LIC to LVEF in TI and TM patients



It can be seen that neither in TI or TM patients is there a relationship between LIC and LVEF% when analysed in cross section. In order to understand the relationship between LIC and LVEF however an analysis of changes in individual patients with time in response to chelation therapy is helpful and this is examined in further detail later in this chapter.

4.6.1.4 Relationship of LIC and liver T2* to cardiac T2* in TI and TM

Figure 4.6.1.e: The relationship between 1/T2* and LIC



Legend: all results for patients converted to R2* showing the TI in red squares and TM as black diamonds

There is no relationship between LIC (derived from liver T2*) and R2* in the heart (1/CT2*) in the TI patients or in the TM patients. Both TI and TM patients had been previously treated with chelation therapy, thereby disturbing any relationship which might have existed pre- chelation therapy.

4.6.2 Factors affecting change in cardiac T2* with time

The analyses in section 4.6.1 were cross sectional and did not analyse changes in parameters with time. In this section the effect of intervention with desferrioxamine (DFO) on these same parameters is examined. The improvement or worsening of cardiac T2* with time could in principle be affected by total body iron burden, as estimated by LIC, and/or the effects of chelation therapy. These are examined in sections 4.6.2.2 and 4.6.2.3. Before this, however, it is important to understand whether ferrioxamine could influence the measurements being undertaken. As explained in section 4.2.3, ferrioxamine has been used in the past as a contrast agent with T1 weighed sequences and although this could not have a significant effect on T2 weighted sequences such as the T2*, it was important to know that no major effect of acute chelation with DFO was observed.

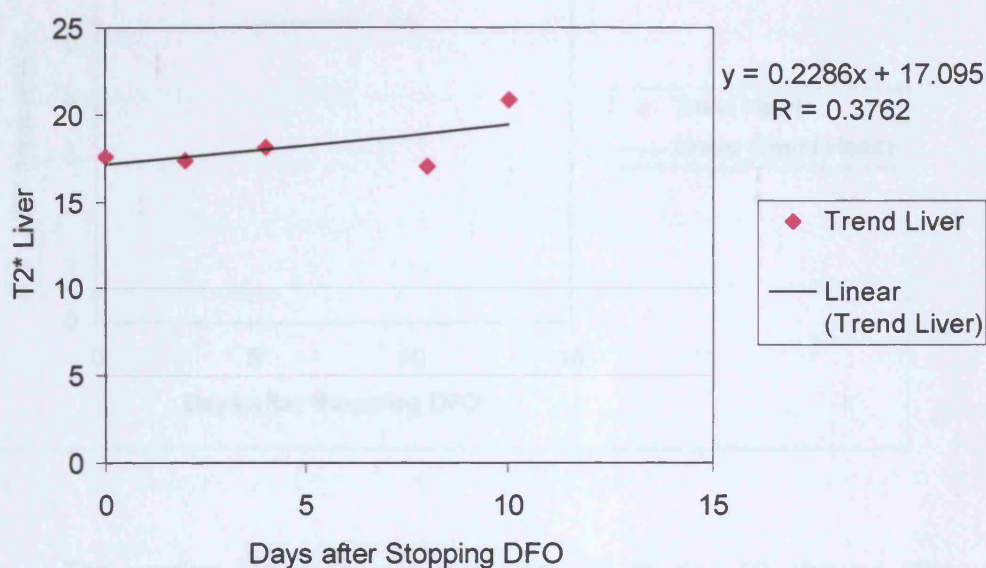
4.6.2.1 *The influence of ferrioxamine on T2* values with DFO chelation.*

Patients follow a variety of practices when attending for CMR assessment. Some attend with the DFO being administered simultaneously; others will not have had DFO for 48 hrs or greater prior to the assessment. The other more important concern is the effect of ferrioxamine in the cellular compartment on the T2* measurement as discussed in section 4.2.3.

One patient underwent sequential CMR on day 0, 2, 4, 8, and 10. The day 0 CMR was with the DFO being administered via a balloon infuser at the time of CMR. The DFO was stopped immediately post CMR assessment and the subsequent CMR measurements were all taken without any DFO. The aim was to see if cardiac and hepatic T2* values would change significantly once the ferrioxamine had cleared from

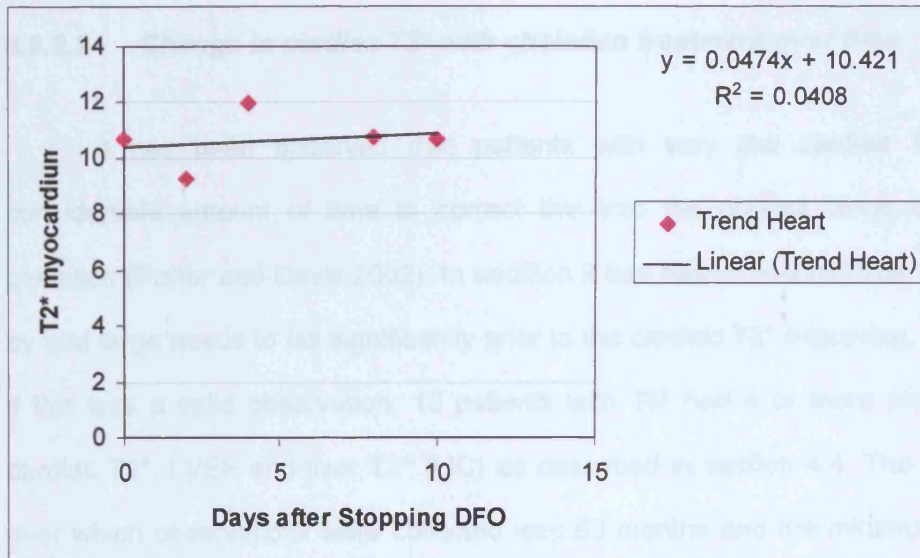
the intracellular compartment and whether DFO being administered at the time of the scan made any difference to the T2* measurement.

Figure 4.6.2.a: T2* of liver at T0 and then 48 hourly intervals post DFO infusion



There was some correlation between the liver T2* and time off chelation but the difference was of little clinical relevance because the liver iron values changed from 0.67 mg/g/dw to 0.56 mg/g/dw. This patient had a low hepatic iron burden, but it is possible in a heavily iron loaded patient the value would alter significantly.

Figure 4.6.2.b: Cardiac T2* at T0 and then up to 10day post DFO infusion



The cardiac T2* measurements from T0 to day 10 showed little variation returning an R=0.2. However on further analysis, the T2* difference between these points is considerable changing from 9.2 ms on day 2 to 11.9 ms on day 4.

These differences are in keeping with our observations of urinary iron excretion of ferrioxamine in patients on DFO infusions where ferrioxamine excretion was seen for at least 10 hours after cessation of DFO infusion. This is also very similar to the classical study by Roberts and Bomford (Roberts and Bomford 1988) who demonstrated that ferrioxamine egress from the intracellular compartment to the extracellular was initially rapid but then much slower once equilibrium is reached between the extracellular and intracellular compartments at 24 hours (Roberts and Bomford 1988).

Although ferrioxamine may play a role in altering the T2* it is unlikely to change a normal T2* value into a severe loading one (conversion of a level of >20 ms to <8 ms)

and provided that patients follow a similar protocols should not be the cause for great deviation in the values.

4.6.2.2 *Change in cardiac T2* with chelation treatment over time*

It has been observed that patients with very low cardiac T2* it takes a considerable amount of time to correct this into the normal range despite optimal chelation (Porter and Davis 2002). In addition it has been observed that the hepatic iron by and large needs to fall significantly prior to the cardiac T2* improving. In order to see if this was a valid observation, 13 patients with TM had 4 or more measurements of cardiac T2*, LVEF and liver T2* (LIC) as described in section 4.4. The maximum time over which observations were collected was 60 months and the minimum time was 12 months. All patients were treated with standard DFO regimes according to their ferritin and therapeutic index as described below. Table 4.6.2.2 shows the chelation characteristics of the patients and the mean rate of improvement of the cardiac T2*. Compliance was estimated from patient's interviews in clinic.

The mean rate of change of cardiac T2* was calculated by dividing the change in T2* between consecutive observations by the number of months between those scans. This gave the change in T2* per month between consecutive scans. All the individual change in T2* per month results were calculated for each patient and this data was used to derive figure 4.6.2e. In individual patients the mean of the change in T2* per month were calculated.

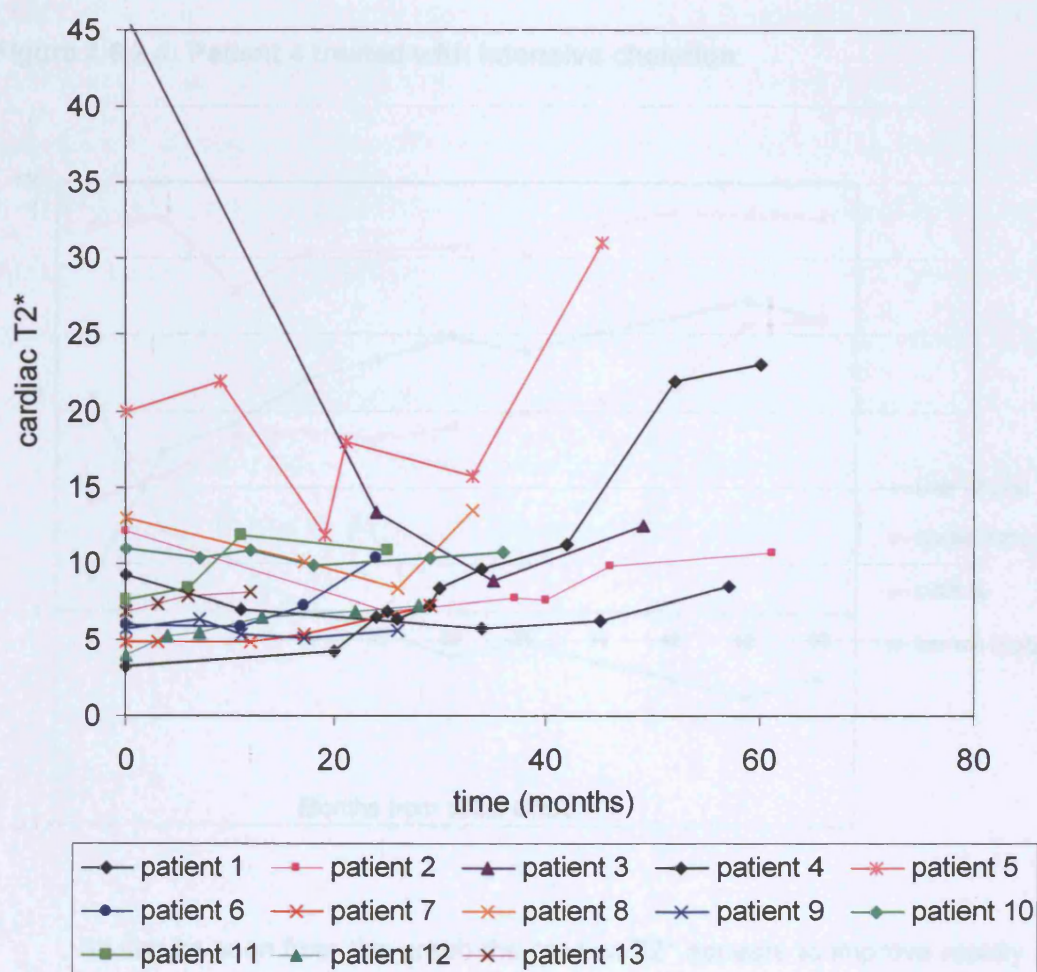
Table 4.6.2.2 Change in cardiac T2* with chelation treatment over time

patient	diagnosis	chelation	Duration in hours per day	days	route	compliance following initial scan	liver iron (mg/g/dw)	lowest cardiac T2*(ms)	mean rate of change of cardiac T2*/month
1	TM	DFO	24	7	IV	50%	11.3	5.7	0.12
2	TM	DFO	24	7	IV	75%	13.2	6.8	0.22
3	TM	DFO	24	7	IV	75%	7	8.9	0.26
4	TM	DFO	24	7	SC	100%	10	3.2	0.37
5	TM	DFO/L1	24	6	SC	100%	4.6	11.8	1.38
6	TM	DFO/L1	12	5	SC	50-75%	5.9	5.9	0.33
7	TM	DFO	24	7	IV	100%	14	4.9	0.06
8	TM	DFO	24	7	IV	100%	1.3	8.4	0.12
9	TM	DFO	12	5.5	SC	100%	15	5.7	-0.04
10	TM	DFO	12	5	SC	100%	2.1	10.3	0.05
11	TM	DFO	24	6	SC	100%	0.6	7.6	0.26
12	TM	DFO	24	7	IV	100%	9.3	4	0.13
13	TI	DFO	24	7	IV	50%	3.5	6.9	0.12

The mean rate of change of cardiac T2* was calculated for individual patients in this table. In section 4.6.2.3 the rate of change in cardiac T2* shall be assessed for all patients analysed above (1 to 13) according to the severity of the myocardial iron load. As can be seen from figure 4.6.2.c, and table 4.6.2.2 the rate of change is extremely variable in patients with some showing rapid improvement and others less so. The reasons for this will be discussed in more detail in section 4.6.2.3.

Figure 4.6.2c shows the change in cardiac T2* over time in the 13 patients and is plotted from sequential CMR results for the individual patients.

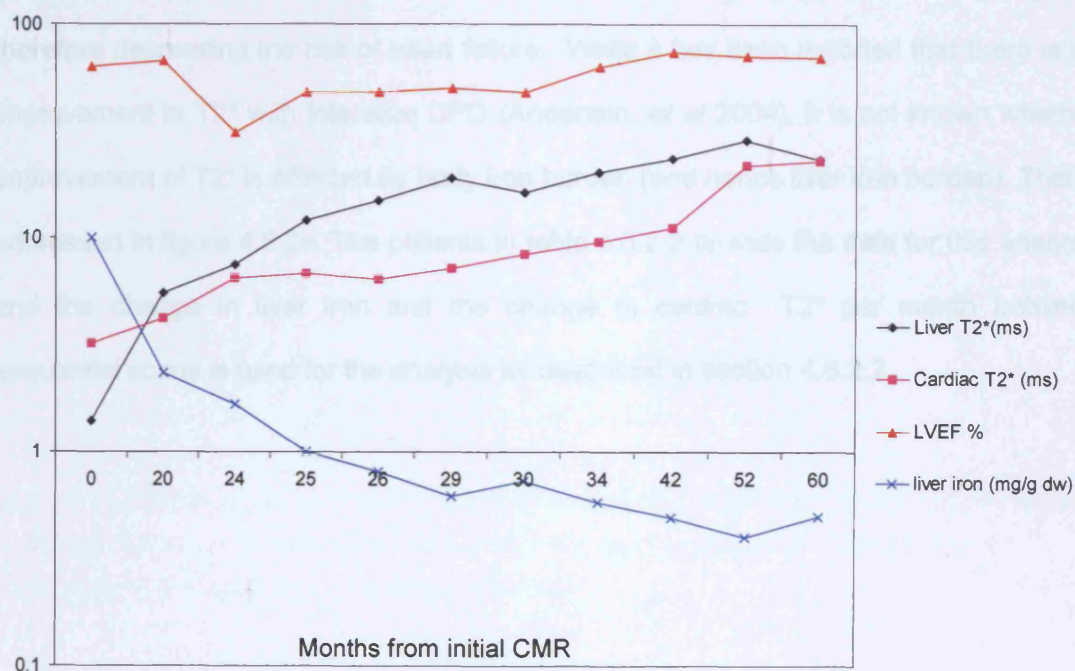
Figure 4.6.2.c: Changes in cardiac T2* over time in patients 1 to 13



Patient 4 is shown in more detail in figure 4.6.2d. He was initiated on an intensive SC DFO regime at 60 mg/kg/day 24 hr infusions following a CMR at T0. The adherence to therapy was optimal but he developed cardiac decompensation thought to be secondary to an episode of myocarditis. IV chelation was initiated and 4 weeks later the ejection fraction had improved significantly. He remained on this regime

subcutaneously until 42 months from first CMR when he started on combination treatment with Deferiprone on 7 days a week at a dose of 75mg/kg/day along with desferrioxamine at 40mg/kg/day on 5 days a week as 24 hr infusers.

Figure 4.6.2.d: Patient 4 treated with intensive chelation.



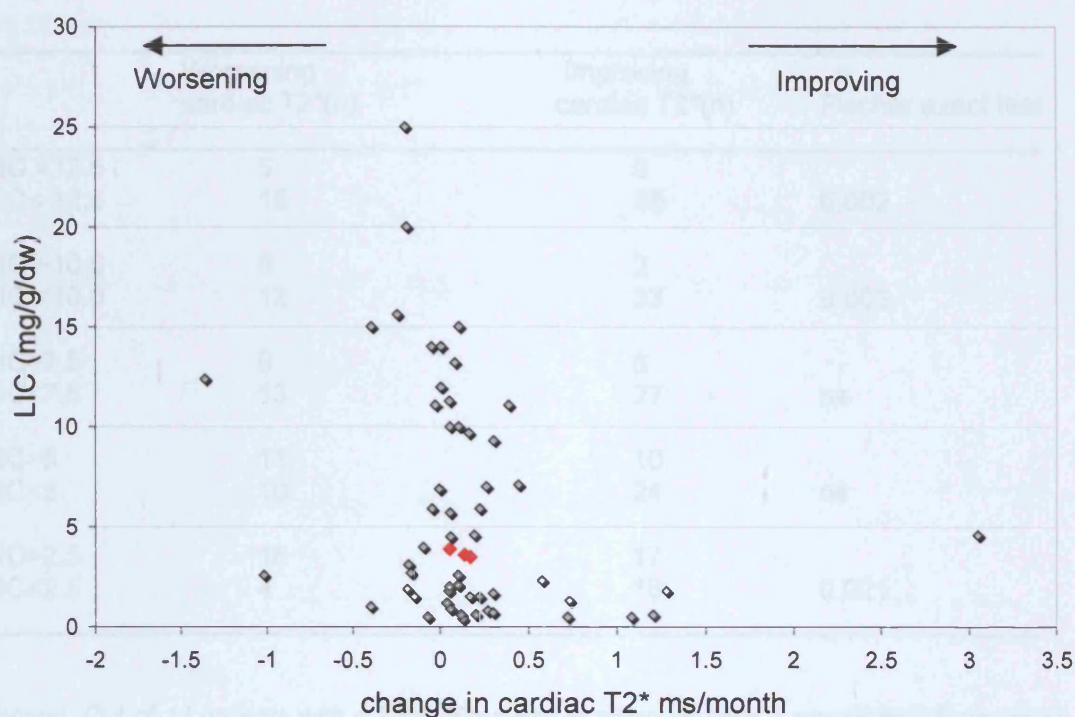
As can be seen from this graph the cardiac T2* appears to improve rapidly once the T2* improves above 10ms and this may be due compliance and dosage of chelator used.

While differences in compliance with treatment could explain some variability in response seen in table 4.6.2.2, it was decided to examine how trends in LIC and absolute LIC values might influence response as this has not been examined previously.

4.6.2.3 *Effect of hepatic iron burdens on response of cardiac T2**

Patients with impaired LVEF have often been treated with intensification of DFO therapy with good results in those who comply with such treatment (Davis and Porter 2000). Due to the increased risk of worsening LVEF in patients with T2* less than 20ms (Anderson, *et al* 2001) in principle intensification of treatment could be applied to patients with shortened cardiac T2* with a view to reducing myocardial iron loading and therefore decreasing the risk of heart failure. While it has been reported that there is an improvement in T2* with intensive DFO (Anderson, *et al* 2004), it is not known whether improvement of T2* is affected by body iron burden (and hence liver iron burden). This is addressed in figure 4.6.2e. The patients in table 4.6.2.2 provide the data for this analysis and the change in liver iron and the change in cardiac T2* per month between sequential scans is used for the analysis as described in section 4.6.2.2

Figure 4.6.2.e: The relationship between liver iron and change in myocardial T2*



Legend: change in cardiac T2 per month plotted against the hepatic iron. The rate of change was calculated for severe, moderate and mild cardiac iron loading according to the cardiac T2* values*
Black squares TM patients; Red diamond T1 patient

In Figure 4.6.2e, it can be seen that improvement in myocardial T2* is more likely when LIC values were < 10 mg/g dry wt. Statistical analysis was performed on these data and these are presented in table 4.6.2.3.

Table 4.6.2.3: The relationship between liver iron and change in myocardial T2*

	Worsening cardiac T2*(n)	Improving cardiac T2*(n)	p Fischer exact test
LIC >12.5	5	0	0.002
LIC < 12.5	15	35	
LIC >10.0	8	2	0.002
LIC <10.0	12	33	
LIC >7.5	9	6	ns
LIC <7.5	13	27	
LIC >5	11	10	ns
LIC <5	10	24	
LIC >2.5	16	17	0.021
LIC <2.5	4	18	

Legend. Out of 13 patients with a T2* < 20ms and in whom at least 4 sequential observations of liver and heart T2* were made, the change in cardiac T2* was calculated (expressed as change in ms/ month) and compared with the mean LIC over that period of observation. The table is analysed from Figure 4.6.2.e and shown the number of observations with worsening (T2* shortening) or improving (T2* lengthening) over the period of observation in relation to LIC values greater or less than those shown. Total number of observations n=55

An LIC > 10 or >12.5mg/g dry wt is associated with a significantly increased risk of the cardiac T2* worsening. A LIC <2.5mg/g dry wt is associated with a significant decreased probability of the myocardial T2* worsening.

4.7 Iron mediated morbidity, T2* and NTBI at matched ages and levels of iron loading.

In order to see whether absolute levels of body iron, as estimated by LIC, were predictive of iron redistribution to the heart or elsewhere and whether this effect was the

same for thalassaemia and sickle syndromes, patients with SCA or TM who had all had cardiac and liver MRI and matched for age and liver iron levels, were studied. 26 patients with SCA who had a history of multiple blood transfusions were matched for liver iron and age with 26 TM patients. Patient characteristics were as described in section 4.4. Complications of iron overload, heart iron (T2*) and plasma NTBI and transferrin saturation were then compared between the sickle and thalassaemia groups.

4.7.1 Characteristics of the two cohorts.

Table 4.7.1.1: Baseline characteristics of the two cohorts

Variable	sickle cell group (n=26)	Thalassaemia group (n=26)	P value
Age (years)	34 \pm 12	29 \pm 8	
Gender (males)	19	15	
Serum ferritin (μ g/l)	1806 \pm 2079	2032 \pm 1908	0.59
Units transfused	191 \pm 172	828 \pm 368	<0.00001
Cumulative iron load 1	25.2 \pm 25.5	219 \pm 120	<0.00001
Chelation therapy with DFO	5	26	
Splenectomy	N/a	11	

Legend Patient characteristics: ¹For the calculations of cumulative iron load the units of blood venesected was accounted for in the calculations but chelation therapy was not. This is described in section 4.4

Table 4.7.1.2: LIC, cardiac T2*, NTBI and transferrin saturation for all patients

Patient set	Liver iron mg/g dw		Cardiac T2* ms		NTBI $\mu\text{mol/l}$		Transferrin Saturation %	
	SCA	TM	SCA	TM	SCA	TM	SCA	TM
1	9.8	9.3	29.1	11.3	1.92	0.85	80	95
2	0.5	0.9	39	27.9	-1.08	4.26	20	85
3	1.9	1.5	30	22.4	-0.68	0.18	38	96
4Y	20	20	22.4	9.2	3.77	5.01	90	100
5Y	8.4	8	33.8	22.9	0.89	5.75	90	100
6	1.95	1.9	56	14.1	3.3	2.2	80	75
7	2.25	2.4	26.6	5.6	-0.53	4.48	70	70
8Y	8.8	8.2	33.2	38.3	1.2	4.3	70	85
9	1.2	1.3	34.2	29.3	-0.6	0.4	40	55
10	15.9	13.3	53.2	6.8	-0.9	3.94	20	96
11	1.4	1.7	37.7	17.8	0.8	-0.3	85	45
12Y	5.7	4.2	29.3	56.8	-0.8	1.72	30	60
13	0.53	0.6	37.6	30.1	-1.2	4.7	27	100
14	1.6	1.9	41.5	7.2	-0.96	5.2	35	100
15	10	9.6	35.9	9.6	5.58	4.64	100	100
16	0.46	0.6	37.7	9.6	-0.94	6.61	37	100
17	0.46	0.5	36.1	27.9	-1.04	3.78	41	99
18	4.45	3.8	36.6	18.9	2.51	4.26	100	96
19	0.75	1.8	41.7	10.2	-0.83	6.4	40	95
20	0.9	0.9	25.4	10.9	-0.35	7.28	47	100
21Y	8.15	7.7	27.8	7.3	-0.67	-1.9	47	95
22	2.9	3.1	37.6	17.9	-0.95	4.4	30	100
23	3	4	25.6	28.8	-0.63	-0.9	37	43
24	0.5	1	24.6	10.7	-0.98	3.8	30	100
25	0.4	0.9	26.7	20.7	-1.16	3.5	25	100
26	1	0.7	33.1	13.5	-1.22	5.9	33	100
mean	4.3	4.2	34.3	18.7	0.17	3.5	51.6	88.1
median	1.9	1.9	34.0	15.9	-0.68	4.3	40.0	96.0
P value	0.42*		0.00002^		0.00001#		0.00001~	

Legend: 26 patients with SCA or TM matched for liver iron are shown:

**: liver iron well matched between SCA and TM patients.*

^: mean cardiac T2 in SCA patients 34.3 ms but in TM is lower at 18.7 ms.*

#: significant difference between NTBI in SCA and TM

~: significant difference in transferrin saturation between 2 cohorts.

□: Sick cell patients who were on chelation with DFO

Blue text: Sick cell anaemia patients with high liver iron load but no NTBI and unsaturated transferrin

4.7.2 Complications in Sickle cell anaemia and thalassaemia major

It has been observed that complications secondary to iron overload appear less frequently in patients with SCA. To see if this was the case patients were matched for liver iron burden because the hepatic iron level is a good reflection of total body iron as discussed earlier. Table 4.7.2.1 shows the frequency of complications from iron burden.

Table 4.7.2.1: Complications found in SCA and TM cohorts

variable	sickle cell	thalassaemia
hypothyroid	0	2
hypogonadotrophic hypogonadism	1*	18
hypoparathyroidism	0	5
diabetes mellitus	1^	7
cardiac history	3 #	10 ~

Legend: *: sickle patient aged 35yrs with premature ovarian dysfunction and intermittent hypogonadal indices over the course of 6 months.

^: non insulin dependant diabetes in patient above 45 years of age and low iron burden.

#: 1 inferior wall myocardial infarction, 1 small vessel disease documented on cardiac catheter and third patient had Atrial Fibrillation requiring medication. All above 45 years of age at diagnosis.

~: Paroxysmal Atrial fibrillation n=4; cardiac failure n=6; All 10 patients below 45 yr.

Table 4.7.2.1 shows clearly that complications that are classically seen secondary to iron overload are seen less frequently in SCA with only five out of twenty

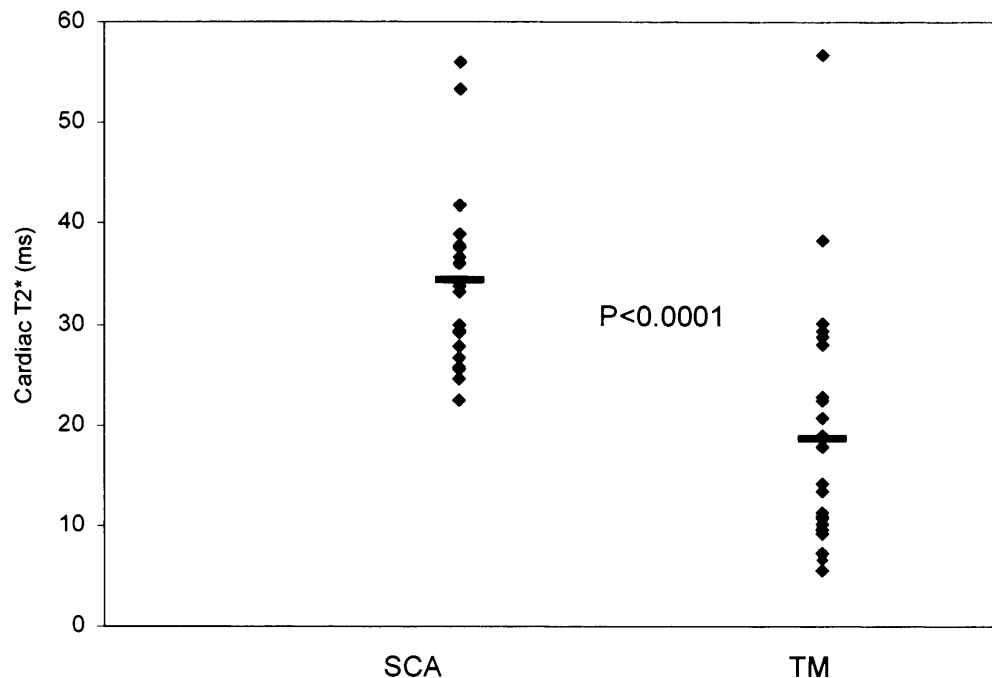
six having a complication and from these only one may have been related to iron burden the others were probably age associated complications.

For the following analyses in section 4.7.3 data is taken from table 4.7.1.2.

4.7.3 Myocardial T2* in SCA and TM matched for LIC

The cardiac complications, which are the most serious manifestations of iron overload, were not seen in the patients with SCA. The most striking finding when matching these patients for hepatic iron burden was the absence of cardiac iron loading as measured by T2* in the SCA group (Figure 4.7.3a). There is a statistically significant higher myocardial T2* in patients with SCA ($34.3 \text{ ms} \pm 8.1\text{ms}$), median 34ms compared to TM ($18.7 \text{ ms} \pm 11.9\text{ms}$), median 12.4ms, with similar degrees of liver iron loading ($p < 0.0001$) suggesting that there was little or no iron deposition in the myocardium in the SCA cohort of patients at similar iron burden

Figure 4.7.3.a: Cardiac T2* in SCA and TM patients matched for iron loading



Legend: P value is for the T2 of SCA versus the TM patients using student T test.*

The reasons why cardiac iron loading differs between SCA and TM patients could have several explanations. Firstly the duration of transfusion differs significantly between the SCA and TM patients. The TM cohort began regular transfusion regimes within the first few years of life whereas the SCA patients did not. If on regular transfusion programmes, SCA patients tend to have exchange transfusions. In this way the cumulative iron burden and the duration of exposure to high levels of iron overload is less even though the liver iron measurements at time of analysis are similar. A second explanation is that the TM patients have been treated with DFO for many years. Patients born before the late 1970s were started with chelation therapy in late childhood or adolescence and TM children born after this time were generally started at 2-3 years of

age on DFO. As this drug appears to have preferential iron removing effects from the liver compared with the heart (Anderson, *et al* 2004), then the ratio of heart to liver iron would be perturbed by such treatment; and this effect which would be relatively lacking in the SCA patients who received relatively little DFO (n=5).

Another important mechanism to consider, as outlined in the introduction is that in some way iron is 'withheld' in macrophages SCA, even at high levels of iron loading, leading to a lower transferrin saturation and NTBI. In order to address this question, these variables were measured in the same 26 patients in the TM and SCA groups.

4.7.4 Plasma NTBI and transferrin saturation in SCA and TM matched for LIC.

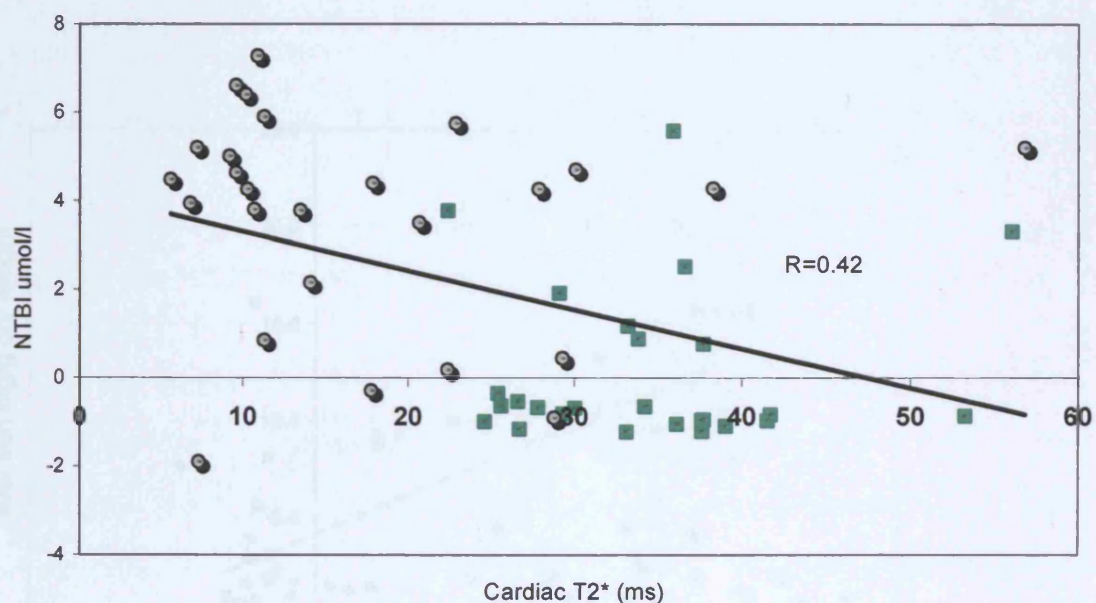
Table 4.7.1.2 shows NTBI values and transferrin saturation for each of the 26 patients in each group. It can be seen that NTBI is significantly higher in the TM (3.48 μ M) than in matched SCA patients (0.17 μ M; $p < 0.0001$). These differences parallels lower transferrin saturations in the SCA patients (51.6%) than the TM patients (88.2%; ($p < 0.0001$).

NTBI is generally found at high iron burdens when transferrin is completely saturated. In these patients who were matched for liver iron loading, this difference may be explained by the chronic inflammatory response in SCA and its effect in sequestering iron in the reticuloendothelial cells. It is thought that NTBI is important for the development of complications from iron overload due to the free radical damage and that NTBI is responsible for iron deposition in the intracellular compartment (Link, *et al* 1993, Randell, *et al* 1994). It can be seen from table 4.7.1.2 that NTBI is significantly lower and the cardiac T2* is normal in the SCA patients who have been matched closely for liver iron with TM.

4.7.5 Relationship of NTBI to myocardial T2*

Since the presence of plasma NTBI has been clearly linked to cardiac iron in animal studies (Oudit, *et al* 2003), the relationship between myocardial T2* and NTBI was examined in the TM and SCA (Figure 4.7.5a) It can be seen that if the overall relationship is examined for all patients (Figure 4.7.6a) there is a clear trend of decreasing T2* at higher NTBI values.

Figure 4.7.5.a: Relationship of NTBI to myocardial T2*



Legend: Relationship between NTBI and cardiac T2* in SCA and TM patients matched for hepatic iron. Green Squares: SCA ; TM: black diamonds

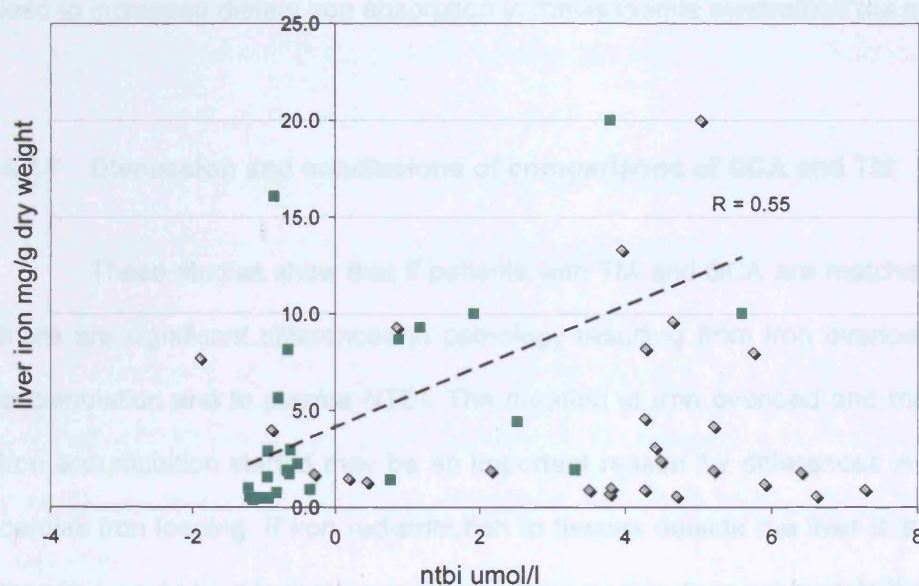
R is for all patients as a single group=0.42; P=0.002

However combining two different groups of patients may confound this analysis. When the relationship of NTBI to cardiac T2* is examined for SCA patients only there is

no correlation ($R=0.08$; $P=0.69$) and for the TM this is only ($R=0.11$; $P=0.69$). Thus within groups NTBI values do not predict or assist with evidence of cardiac iron loading suggesting that the apparent association between NTBI and cardiac $T2^*$ (Figure 4.7.5a) may be the result of association rather than causation. In other words two things which are increased more in TM than in SCA are being compared, but they may not be causally related.

4.7.6 Relationship of liver iron to NTBI

Figure 4.7.6.a: Relationship of LIC to NTBI in patients matched for liver iron



Legend: Relationship between NTBI and hepatic iron in SCA and TM patients matched for hepatic iron. $R=0.55$ $P=0.004$ in SCA. The dotted trend line is the R for SCA.

Green Squares: SCA ; TM: black diamonds

It can be seen that when SCA and TM patients are analysed together that there is no overall relationship between LIC and NTBI ($R=0.17$; $P=0.23$; $n=26$). When the two groups are analysed separately, there remains a trend of increasing NTBI with increasing LIC in SCA ($R=0.55$; $P=0.004$; $n=26$), but is not seen for the TM patients ($R=0.029$; $P=0.88$; $n=26$).

This observation is of interest when considered in relation to Chapter 3 where it was found in young thalassaemia patients that NTBI was present even before iron loading. This relative independence of NTBI from body iron loading in thalassaemia syndromes compared with sickle syndromes may relate to the ineffective erythropoiesis seen in thalassaemia compared with the haemolysis which is the main cause of anaemia in SCA. This difference is also likely to be important in the mechanisms, which lead to increased dietary iron absorption in thalassaemia syndromes but not in SCA.

4.7.7 Discussion and conclusions of comparisons of SCA and TM

These studies show that if patients with TM and SCA are matched for liver iron, there are significant differences in pathology resulting from iron overload, cardiac iron accumulation and to plasma NTBI. The duration of iron overload and the age at which iron accumulation started may be an important reason for differences in pathology and cardiac iron loading. If iron redistribution to tissues outside the liver is a slow process, then this would be a logical explanation. However this does not explain the differences in NTBI and transferrin saturation in the patients matched for iron loading. As discussed in section 4.1 the chronic inflammatory state with raised type II cytokines such as IL6 as well as increased IL1 β and IL8, would be expected to decrease transferrin saturation though the hepcidin mechanism (see chapter 5) and this would explain the lower NTBI in

SCA at matched levels of iron. A second explanation for the lower transferrin saturation and NTBI in SCA patients could be that ineffective erythropoiesis is a driving factor for NTBI formation and that this is relatively lacking in SCA compared with thalassaemia syndromes. Since there appears to be a relationship between NTBI and cardiac T2*, this supports the notion lower NTBI at matched LIC in SCA patients could lead to decreased secondary iron redistribution, even at matched levels of transfusional overload. In order to answer this question conclusively a prospective study of iron distribution comparing children with thalassaemia and sickle syndromes commenced on transfusion would be required.

4.8 Relationship between Cardiac T2* and Iron burden in Thalassaemia

Intermedia

Iron overload in TI occurs due to a combination of factors, which makes it difficult to compare them to TM or SCA. Unlike SCA, iron absorption is increased, probably because of the greater degree of ineffective erythropoiesis in TI. The rate of iron accumulation dietary iron is variable, presumably depending on the extent of ineffective erythropoiesis.

Definitions for TI was as outlined in section 4.4 thirteen patients with TI underwent CMR assessments and also had NTBI measurements. Patient characteristics were as described in section 4.4. Table 4.8.1.1 shows the baseline characteristics of this group.

Table 4.8.1.1: Patient characteristics with thalassaemia intermedia

Variable	Thalassaemia intermedia (n=13)
Age (years)	34± 10
Gender (males)	7
Serum ferritin (ng/ml)	1384±1320
Units transfused	129 ± 164
Chelation therapy with DFO	8

It can be seen that the TI patients received less blood and had lower ferritin levels compared to the SCA and TM cohorts discussed earlier.

Table 4.8.1.2: Complications in TI patients

variable	Number of affected patients
hypothyroid	0
hypogonadotrophic hypogonadism	0
hypoparathyroidism	0
diabetes mellitus	0
cardiac history	2#

Legend: #: 1 rheumatic mitral valve disease;1 pulmonary hypertension

Table 4.8.1.3: Liver iron cardiac T2* and LVEF and NTBI for TI patients

patient	units transfused	liver iron mg/g/dw	cardiac T2*	EF%	ferritin ng/ml	NTBI umol/l
1	209	9.8	30.0	77	653	3.99
2*	210	9.1	37.2	56	2303	2.64
3*	79	8.5	46.7	66	243	4.93
4	589	5.6	8.1	70	3814	2.97
5	72	7.7	54.0	74	725	2.65
6	72	5.6	68.0	59	396	1.38
7	0	3.5	25.7	72	476	3.38
8	0	3.2	43.0	78	414	-0.44
9	209	10.9	12.3	73	3322	0.77
10	210	10.0	28.1	66	1815	4.97
11^	10	10.9	34.9	59	3120	3.00
12	0	1.7	39.5	65	152	-0.51
13^	14	9.6	34.2	56	564	1.38
mean	128.8	7.4	35.5	67	1384	2.39
SD	164.3	3.1	16.0	8	1320	1.79
SEM	45.6	0.9	4.4	2.1	366.1	0.5

Legend: *: cardiac complications (pulmonary hypertension and rheumatic mitral valve disease)

^: E β thalassaemia

It can be seen that transfusion requirements are quite varied amongst the patients but some patients have significant iron overload as measured by CMR even when transfusion frequency had been minimal. On further analysis no relationship was found between NTBI and cardiac T2* or NTBI and units of blood transfused. However there was a relationship between units of blood transfused and cardiac T2*

Iron loading in TI is multi-factorial and increased gastrointestinal iron absorption is an important mechanism. These patients are often transfused at various points in their lives such as during intercurrent infections, pregnancy, symptomatic anaemia, to aid

healing of venous ulcers, and to reduce extramedullary haematopoiesis. Patients with TI develop cardiac iron loading similar to the TM patients in the third or fourth decade (Pippard and Weatherall 1984) and infrequent transfusions will enhance this rate of iron loading. As can be seen from table 4.8.1.3 some TI patients have a high liver iron even when they have had minimal transfusion and tend to have more severe anaemia (mainly E β thalassaemia) thereby supporting the role of increased gastrointestinal iron absorption. Interestingly patients 11 and 13 were part of the pro-hepcidin TI cohort and both had very low levels of serum pro-hepcidin (46 and 60 ng/ml respectively).

4.9 Conclusions

Myocardial iron loading is an important cause for premature death in transfused patients. To date there is no consensus on how best to quantify this with significant limitations to endomyocardial biopsies and the limited availability of tissue validation data for the different MRI techniques used. In this chapter cardiac T2* has been used as a means of quantifying cardiac iron loading. In the absence of suitable tissue validation the data presented supports the measurement of T2* as an important prognostic indicator with a good correlation below 20ms with LVEF.

Recent publications using this technique have shown no correlation with liver iron and documented that patients with low liver iron may have a low T2* (Anderson, *et al* 2001).

The importance of longitudinal monitoring of patients has been shown in this chapter in patients with transfusion associated iron overload. Clearance of myocardial iron is slow, particularly when there is a severe degree of hepatic loading in patients. However,

once the liver iron falls significantly then the cardiac T2* improves into the moderate range.

Concern about ferrioxamine altering the cardiac T2* need to be further addressed. In our study we only performed sequential monitoring in one patient. The degree of change in the T2* over a period of 48 to 84 hours was significant in this patient. On this basis, until further data is available on more patients, it is probably best to advise patients to have the CMR assessment at the same point in their chelation cycle on each occasion i.e. if they normally have the CMR assessment whilst they have their DFO infusion, then future CMR assessments should also be undertaken with the DFO infusion being administered. This would remove any variability due to slow Ferrioxamine clearance from the cells.

CMR (with simultaneous MRI of hepatic iron) assessment is very valuable in SCA for assessing the cardiac and liver iron stores. It is apparent that patients with SCA do not develop as rapid or as severe iron loading as TM patients. This is because the transfusions are predominantly in the form of exchange transfusions with iron being lost from the venesected units, in addition, the transfusions are also less frequent, often being undertaken intermittently with urgent transfusion being performed for sickle complications. There does appear to be a relationship between cardiac T2* and NTBI when all patients (SCA and TM) are analysed as a group.

Damage to the myocardium is related to NTBI and free radical damage associated with this. Patients with SCA tend to have much lower levels of NTBI at matched levels of iron burden compared to TM patients. The NTBI present in sickle patients and in TM adult patients will predominantly be due to excess iron secondary to transfusion rather than due to ineffective erythropoiesis. It has been shown in SCA that even at steady state there are increased inflammatory markers and cytokines such as IL1, IL6, IL8 and TNF (Makis, *et al* 2000a). These cytokines may down regulate the

levels of NTBI at matched levels of iron loading by causing sequestering of iron in the reticuloendothelial system.

The TI cohort are transfused less frequently than TM patients but they are more prone to develop iron loading compared to the SCA patients because of increased gastrointestinal iron absorption. The reasons for this difference will be discussed in more detail in chapter 5.

CMR is a very valuable tool in the management of iron overload and should be offered to all patients on regular transfusion regimes. It has provided a great deal of information on the distribution of iron in the body, this has lead to improved management of patients by the use of intensification of chelation in those with severe myocardial iron loading. Although tissue validation of T2* as a methodology is still awaited, it nevertheless measures a clinically important parameter and has prognostic significance.

Chapter Five

Hepcidin metabolism, iron overload and NTBI in thalassaemia and sickle disorders

5.1 Introduction

In humans, there is no regulated excretory mechanism for iron, with the only means of removal of iron being shedding of the gastrointestinal mucosal cells and skin cells at a rate of about 1-2 mg per day. Body iron is therefore usually regulated by the amount of iron absorbed from food. Over the last few years, several new regulators of iron absorption have been identified. Prominent amongst these has been the discovery of hepcidin which is a 25 amino acid peptide expressed in hepatocytes and first isolated from human urine by Park et al (Park, *et al* 2001). This molecule appears to inhibit absorption of iron from the enterocyte and iron release from macrophages.

5.1.1 Structure, function and tissue expression of hepcidin

Pigeon et al (Pigeon, *et al* 2001) found that in carbonyl iron loaded mice, a 225 base pair cDNA (HEPC1) was over expressed and when the murine full length cDNA was isolated, it was found to encode for an 83 AA protein with close homology of its C terminus with the human hepcidin isolated in urine by Park et al (Park, *et al* 2001). It was found that the human HEPC1 gene on chromosome 19 showed close homology to the mouse HEPC1 with 3 exons and 2 introns as well as a USF2 (upstream stimulating

factor 2) located at the 5' end. It encoded for an 84 amino acid protein, which showed a 54% homology to mouse pro-hepcidin (Kulaksiz, *et al* 2004).

Hepcidin was found to have antimicrobial activity and its relationship to iron metabolism was not noted by Park *et al* but was first recognised by two other groups simultaneously; Nicholas *et al* and Pigeon *et al*. Nicholas *et al* (Nicolas, *et al* 2001) found that *Usp2* knockout mice progressively developed multi-organ iron overload. Using subtractive suppressive hybridisation techniques with these mice and wild type mice they isolated cDNA encoding for hepcidin. Hepcidin was found to be absent in *Usp2* knockout mice and in a second publication using transgenic mice that constitutively over-express hepcidin, it was noted that these animals died of severe iron deficiency anaemia within a few hours of birth (Nicolas, *et al* 2002a). Hepcidin was therefore described by Nicolas *et al* as a negative regulator of iron absorption.

Pigeon (Pigeon, *et al* 2001) whilst working on mice fed iron rich and iron poor diets found that hepcidin mRNA increased with the degree of iron loading and decreased in iron deficient mice. In murine models hepcidin mRNA expression was primarily in the liver with only weak expression in the stomach, intestine, colon, lungs, heart and thymus. In human tissues, expression of the mRNA was also strong in the liver but significant expression of hepcidin mRNA was also seen in the left atrium and in the spinal cord (Pigeon, *et al* 2001)

Pigeon found that the pro-hepcidin localised to the nucleus and if this was masked it localised to the cytoplasm, it was therefore postulated that pro-hepcidin may undergo cleavage in the hepatocytes prior to excretion of the mature 25 AA form and the other smaller forms of hepcidin. Recent work by Wallace *et al* (Wallace, *et al* 2005) has resulted in the generation of a recombinant mouse pro-hepcidin molecule of 7.5 kDa which localised to the secretory intracellular pathway and further work is

planned to examine the post Golgi processing of pro-hepcidin and its response to stimulants such as IL6.

Kulaksiz et al (Kulaksiz, *et al* 2004) generated antibodies to the mid portion and C terminus of the pro-hepcidin and developed an ELISA assay to look at the presence of pro-hepcidin in human sera. It was shown that the antibody identified a single 10 kDalton band that co-migrated exactly with immunoreactive hepcidin in tissue extracts of liver and HepG2 cells.

5.1.2 Effect of anaemia and hypoxia on hepcidin metabolism

The relationship of hepcidin expression to anaemia was noted by Nicholas et al (Nicolas, *et al* 2002a) when transgenic mice over expressing hepcidin died of severe iron deficiency anaemia soon after birth. The relation between anaemia and hepcidin was further defined by Nicholas when mice were subjected to anaemia by repeated phlebotomies to induce iron deficiency or exposed to phenylhydrazine. The phlebotomised mice were found to have an 80% decrease in hepcidin mRNA even in the presence of normal liver iron stores and the haemolytic anaemia mouse model was found to have a 3 fold reduction of hepcidin mRNA in the presence of an increased liver and serum iron (Nicolas, *et al* 2002b). It was concluded that hypoxia may be overriding the liver iron mediated down regulation of hepcidin and therefore the role of hypoxia was assessed in mice housed in hypobaric hypoxia chambers. A marked down regulation of hepcidin was seen after 2 to 4 days of hypoxia. Tissue hypoxia secondary to anaemia may play a major role in hepcidin regulation and result in increased iron absorption. Recently Ezah et al showed that samples taken from patients with SCA during steady state did not have abnormal levels of pro-hepcidin (Ezeh, *et al* 2005). A number of

studies have assessed hepcidin or pro-hepcidin in anaemia of chronic disease and found a good association of this with ferritin (Dallaglio, *et al* 2003, Nemeth, *et al* 2003)

5.1.3 Effect of inflammation on hepcidin metabolism

This observation raised interesting questions as to the role of hepcidin in anaemia associated with inflammatory states. Anaemia of chronic disease is characterised by increased reticuloendothelial (RE) iron storage and decreased iron absorption in the face of the anaemia. Park *et al* (Park, *et al* 2001) first noted its relationship to inflammation when one of the patients in whom the hepcidin was isolated developed an infection and hepcidin increased 100 fold in urine samples taken during the acute inflammatory state. In addition Pigeon *et al* (Pigeon, *et al* 2001) showed that hepcidin expression was upregulated by lipopolysaccharide (LPS) in wild type mice by 4 fold and 7 fold in mouse hepatocyte cell culture. Further work on the role of inflammation on hepcidin levels by Nicholas *et al* (Nicolas, *et al* 2002b) using turpentine injections to induce inflammation in mice found that hepcidin mRNA increased 6 fold within 16 hrs of injection. Nemeth (Nemeth, *et al* 2003) carried out confirmatory work on the role of inflammation in stimulation of hepcidin on hepatocytes that were exposed to IL1 α , TNF α and IL6, and confirmed that hepcidin is a type II acute phase reactant with upregulation in response to stimulation with IL6 but not with IL1 α or TNF α .

Further work by Rivera *et al* (Rivera, *et al* 2005) showed that when mice were injected with hepcidin, hypoferrinaemia was induced within 4 hours of injection and when hepcidin excreting xenografts were implanted into NOD-SCID mice, the mice developed anaemia associated with increased hepatic iron stores and a low serum iron (anaemia of inflammation/chronic disease). This scenario is consistent with the observation in

anaemia of chronic disease, in which hepcidin expression increases even in the presence of anaemia. The increase in hepcidin in chronic inflammatory conditions would lead to decreased iron absorption from the duodenal enterocyte and reduced release/increased sequestering of RE iron, which would result in low serum iron and transferrin bound iron and the development of anaemia due to functional iron deficiency.

5.1.4 Relationship of iron overload to hepcidin in humans

Iron loading can occur due to iatrogenic or genetic causes. The most common condition causing genetic iron loading is hereditary haemochromatosis (HH) which can broadly be divided into 4 main types; type 1 is predominately associated with the HFE mutation and has a variable penetrance, type 2 is associated with a severe phenotype and presents in young adults, type 3 is associated with mutations in transferrin receptor 2, type 4 is autosomal dominant and tends to be associated with RE iron loading.

HH types 1, 2 and 3 have predominantly non-RE iron loading. Bridle *et al* (Bridle, *et al* 2003), Gehrke (Gehrke, *et al* 2003) and more recent work by Papanikolaou (Papanikolaou, *et al* 2004) have shown reduced hepcidin in these 3 forms of HH. Nemeth suggested (Nemeth, *et al* 2003) that mutations in hepcidin expression may contribute to the iron loading seen in HH and indeed this has been shown with the discovery of 2 new forms of juvenile HH (HH type 2) due to mutations in the hepcidin gene (Biasiotto, *et al* 2004, Roetto, *et al* 2003).

The relationship of hepcidin and iron overload in secondary haemochromatosis in humans is however unknown. Nemeth (Nemeth, *et al* 2003) found that in patients with transfusional iron loading (n=3) urinary hepcidin (uhepcidin) was increased in amount and there was a good correlation between uhepcidin and serum ferritin. However there

remains a paucity of data on what happens to hepcidin in iron secondary iron overload in humans.

The expression of hepcidin is unclear in haemoglobinopathies such as thalassaemia and SCA. Patients with β thalassaemia syndromes present with anaemia at an early age and the severity of the ineffective erythropoiesis and anaemia is a major factor underlying the transfusion requirements. Iron overload develops in those who are transfusion dependant due to iatrogenic iron via the blood, as well as in those who are transfusion independent secondary to increased gastrointestinal iron absorption. The sickle haemoglobinopathies are associated with considerable anaemia but no increase in iron loading from the gastrointestinal tract has been observed, contrary to the iron loading seen in the TI patients who have a similar degree of hypoxia from anaemia. The anaemia of SCA is predominantly driven by haemolysis whereas in TI, ineffective haematopoiesis is much more evident. Thus with TI red cell destruction is significantly greater in the bone marrow than in SCA. These differences could have consequences on iron loading onto transferrin, NTBI formation and the regulation of hepcidin.

Only 2 studies from the same group have looked at the relationship between thalassaemia and hepcidin expression. Adamsky *et al* using a TI mouse that was transfusion independent found reduced liver hepcidin mRNA (Adamsky, *et al* 2004) suggesting that the down regulatory effect of anaemia overrides the up regulatory effect of iron overload. Follow up work from this group showed decreased hepcidin expression in TM mice (Weizer, *et al* 2004). In addition on exposure of human hepatoma HepG2 cells to human thalassaemic sera they found that hepcidin expression was an average of 3 fold lower than that evoked by normal sera. The down regulating effect with thalassaemic sera but not with normal sera suggested that there may be an upstream factor whose levels may increase in the sera in response to ineffective erythropoiesis and that this may override the upregulation of hepcidin in response to iron overload.

Interestingly Nemeth showed that human hepatoma cells have decreased hepcidin mRNA in the presence of NTBI (Nemeth, *et al* 2003) and this is supported by work in our lab (*in preparation Rafique et al*).

Kearney *et al* (Kearney, *et al* 2007) looked at urinary hepcidin in children with congenital anaemias and found that in TM children no significant difference in uhepcidin was seen however the uhepcidin was significantly lower than control patients for both TI and SCA. In the thalassaemia syndromes there was a good correlation with the ferritin and in the sickle syndromes there was an inverse relationship with the erythropoietic drive. No studies have been done on plasma hepcidin/ pro-hepcidin or its relationship with NTBI in patients with these syndromes.

5.1.5 Relationship of urine and liver hepcidin to plasma hepcidin

It is apparent from the discussion above that clinical studies to date have measured either urine hepcidin (Nemeth, *et al* 2003) or hepatic hepcidin mRNA (Detivaud, *et al* 2005) levels as markers for hepcidin expression and examined changes in these in response to iron deficiency, inflammation and other variables. Very little evidence exists about serum hepcidin. Dallalio *et al* (Dallalio, *et al* 2003) measured 'serum hepcidin' levels in anaemic patients and those with abnormal ferritin measurements using western blot analysis, their antibody bound to a 9 kDa molecule where the predicated molecular weight of the 25 AA form of hepcidin is 2.7 kDa. Dallalio found a good positive correlation between the 9 kDa peptide and ferritin but did not find a difference according to the type of anaemia in contrast to Nemeths data (Nemeth, *et al* 2003).

Dallalio postulated that there was a free (urinary Hepcidin) and a bound form (9 kDa peptide). Prior to Dallalio's publication, the changes in plasma levels have been

assumed to be the same as changes in urine but to date no simple reliable measure of plasma hepcidin has been developed so it is not possible at this stage to say whether urine and plasma hepcidin levels truly parallel each other.

5.1.6 Relationship of serum hepcidin to serum pro-hepcidin.

An antibody has been developed however which recognises pro-hepcidin, which is the immediate and larger product of the HEPC1 gene which was first identified by Pigeon (Pigeon, *et al* 2001) in mice on chromosome 7. The product of HEPC1 is an 83 amino acid (mouse) protein, which has close homology between the C terminus and the 25 amino acid mature chain of human hepcidin. It was found that the human HEPC1 gene on chromosome 19 showed close homology to the mouse HEPC1 with 3 exons and 2 introns as well as a USF2 (upstream stimulating factor 2) located at the 5' end. This encoded for an 84 amino acid protein which showed a 54% homology to mouse pro-hepcidin.

Kulaksiz *et al* (Kulaksiz, *et al* 2004) generated antibodies to the mid portion and C terminus of the pro-hepcidin and developed an ELISA assay to look at the presence of pro-hepcidin in human sera. It was shown that the antibody identified a single 10 kDalton band that co-migrated exactly with immunoreactive hepcidin in tissue extracts of liver and HepG2 cells. Results from the pro-hepcidin ELISA on human sera showed that pro-hepcidin was present in reduced amounts in HH sera compared to normal controls (Kulaksiz, *et al* 2004). No information was given in this study on transfusion support that patients may have received. Recently Ezah *et al* showed that samples taken from patients with SCA during steady state did not have abnormal levels of pro-hepcidin (Ezeh, *et al* 2005). No study has been undertaken to assess the relationship between pro-hepcidin and iron overload in haemoglobinopathies.

5.2 Aims and rationale

In this chapter, we explore how iron loading modulates hepcidin expression measured as mRNA in liver biopsies and expression of pro-hepcidin in plasma in sickle and thalassaemia syndromes. The intention is to gain insight into whether the regulation of iron absorption differs between sickle and thalassaemia syndromes, and whether this could account for the tendency for thalassaemia syndromes to hyperabsorb iron from the gut, as well as the higher levels of NTBI compared to the sickle syndromes at matched levels of iron loading, and the different patterns of distribution of iron between the two disorders (chapter 4).

There were two main aims:

Aim 1: What is the relationship between hepcidin expression and iron burden in liver biopsies in thalassaemia syndromes

Liver biopsy samples have been used for many years in patients with thalassaemia syndromes to assess iron burden by the measurement of liver iron concentration (LIC). In principle, these biopsy samples can provide the opportunity to relate hepcidin synthesis measured as hepcidin mRNA to the liver iron in the same block of liver tissue. Ethical approval was therefore requested and obtained from the ethical committee at UCL/UCLH to measure hepcidin mRNA in paraffin blocks previously obtained for measurement of liver iron. For measurement of hepcidin mRNA using liver

biopsies prospectively, written consent was obtained for hepcidin measurement in addition to liver iron.

Aim 2: What is the relationship of pro-hepcidin with anaemia and iron overload in sickle and thalassaemia syndromes

The lack of liver biopsy samples in patients with SCA but the availability of serum samples from patients with TM, TI, and SCA allowed us to examine the relationship between anaemia and iron overload in more depth. The LIC was the measurement obtained simultaneously during CMR assessment. NTBI, Hb, ferritin measurements were from the time of sampling. Liver iron assessment is discussed in more detail in section 5.3.3.

5.2.1 Patients for Aim 1

40 liver blocks had RNA extraction performed. RNA was retrieved in sufficient quantity in 20 samples. Successful RNA extractions were possible on blocks of biopsies from 1993 to 2000. Controls for the first cohort were 5 biopsy samples that had no excess liver iron by Perls' stain, sufficient RNA was extracted in 3 of these. These samples were blinded to us and no data is available on the haemoglobin levels or ferritin levels of these patients. The controls for the second batch were 10 patients all of whom had undergone liver resection for metastatic adenocarcinoma, sufficient RNA was retrieved on normal liver tissue in 7 of these blocks all of which were from clear liver margins sections with no liver tumour present. Perl's stain was undertaken on these control samples to confirm the absence of iron loading. The patients were 14 TM, 4 transfused and 2 non-transfused TI.

Table 5.2.1.1: Patient characteristics for aim 1

patient characteristics	thalassaemia major	thalassaemia intermedia
number	14	6
male sex	8	4
Mean age (range)	25.6 (5.1)	30 (10.55)
transfusion dependent	14	*4
chelation	14	5
HCV Antibody positive	†5	1
HCV RNA positive	3	1
abnormal AST	4	1

Legend:

All TM major patients transfused 3 to 4 weekly.

**transfusion dependant TI on exchange transfusion (2) or top up transfusion (2) at 8 weekly intervals*

† HCV antibody positive indicative of infection in the past, one cleared virus with treatment, one cleared virus spontaneously. TI patient HCV positive due to previous transfusions

5.2.2 Patients for Aim 2

A total of 73 patient samples were analysed comprising 21 patients with TI, 26 with TM and 26 with SCA. TI samples were collected either in clinic or immediately pre exchange and TM samples were collected when they had been off all chelators for 24 to 48 hrs and 2 to 8 days prior to blood transfusion. All samples for the sickle patients who were transfusion dependant were collected at the start of exchange transfusion. Non-transfused sickle patient samples were collected in outpatients clinics. Any results from patients where samples were taken post transfusion were excluded from the analysis. All patients with serum creatinine above 100umol/l, CRP >10 mg/l or HCV RNA positive

were excluded from the analyses. Any patients who were HCV RNA positive were also excluded from the primary analyses (4) but were included in the specific analysis.

After the exclusions 17 patients with TM, 18 patients with TI of whom 15 TI patients were not currently transfused and 15 patients with SCA were included in the final analysis. In the SCA cohort, 12 patients were on exchange transfusions and 3 had previously been transfused. Normal healthy controls (n=10).

Table 5.2.2.1: Patient Characteristics for aim 2

patient characteristics	thalassaemia intermedia*	thalassaemia major~	sickle cell anaemia #
number	18	16	15
male sex	10	7	9
transfusion dependant	3	16	12¶
chelation	4	16	5
ALT>60u/l	2	2	3

Legend: 21 patients with TI and 26 patients each with TM and SCA were analysed. All patients with CRP >10, detectable HCV RNA, creatinine >100umol/l or who donated samples post transfusion were excluded from the final analysis. Final numbers are shown above.

**TI: all patients who require <8 transfusions per annum or are transfusion independent.*

~ TM: all patients with transfusion dependant beta thalassaemia.

SCA: SCA who may or may not require transfusion.

¶ SCA: transfusion dependants are those who are currently on transfusion programmes. Those who are not currently transfused are the remaining patients (3)

5.3 Methods

RNA extraction and preparation of samples as described in Chapter 2 section 2.11.

5.3.1 PCR methodology

This was performed using previously described methodology ((Laftah, *et al* 2004, Leung, *et al* 2005).

1µg of total RNA was used for cDNA synthesis with the Abgene Reverse-iT 1st Strand Synthesis Kit (ABgene, Surrey, UK). RNA concentration and purity were determined by spectrophotometry. The resulting cDNA transcripts of liver mRNA were used for real-time PCR amplification using the Roche Lightcycler (Roche diagnostics, Germany) and QuantiTect SYBR® Green PCR kit (Qiagen, Sussex, UK) according to the manufacturers protocol. Specific primers were designed from the rat sequences for hepcidin and the constitutively expressed gene, actin:

Hepcidin:

FORWARD	CACGAGGGCAGGACAGAAGGCAAG
REVERSE	CAAGGTCATTGCTGGGGTAGGACAG

Actin:

FORWARD	GACGGCCAAGTCATCACTATT
REVERSE	CCACAGGATTCCATACCCAAGA

To quantify hepcidin mRNA expression, standard curves were generated with known amounts of each gene product. A ratio of relative abundance of the hepcidin gene to actin was calculated by the Lightcycler Relative Quantification software version 1.0 (Roche Diagnostics, Germany).

5.3.1.1 Calculation of results

The first set of RNA extraction was relatively poor in amount due to smaller sample size. The second batch of samples analyses as described above provided a greater amount of RNA. To standardise the results between the two batches of samples, we normalised the control results of each batch to 100% and then calculated the relative value as a percentage of the mean of the control for that batch. The results were analysed on the basis of the % of the mean of normal controls for each run. The mean, standard deviation and the standard error of the mean was calculated where relevant. All p values were derived using the Students T tests and were 2 tailed and of equal variance.

All the haemoglobin results were those taken pre-biopsy. The serum ferritin and AST were the mean of the results for 6 months prior to the biopsy.

5.3.2 Liver iron quantification in paraffin blocks

The liver iron was obtained from that biopsy sample by drying followed by acid digestion as per the method described by Barry (Barry 1974). Mr Gareth Ellis in Special Haematology performed these at the Royal Free Hospital. The liver iron concentration was calculated by measuring the optical density at 535nm and using the formula:

$$\text{Iron content of Biopsy } \mu\text{g} = \frac{\text{OD Digest}}{\text{OD Standard}} \times [\text{Fe}]$$

$$\text{Tissue Iron Concentration } \mu\text{g}/100\text{mg Dry weight} = \frac{\text{Iron content of biopsy}}{\text{Dry weight of Biopsy mg}} \times 100$$

5.3.3 Prohepcidin ELISA

Prohepcidin ELISA was performed as described in section 2.10, Chapter 2. The assay was performed at a 1:1 dilution throughout.

5.3.3.1 Calculation of results

The pro-hepcidin value of each sample was obtained as follows:

Using linear-linear paper, a standard curve was constructed by plotting the average absorbance (Y) of each Reference Standard against its corresponding concentration (X) in ng/ml.

The average absorbance of each sample was used to determine the corresponding pro-hepcidin value by simple interpolation from this standard curve, multiplying by the initial sample dilution.

Normal range: 51.6 -153.9 ng/ml; mean 106 (SEM 32.1)ng/ml for the kit.

Normal control values in our lab :56-190 ng/ml; mean 119 (14.3) ng/ml

5.3.4 Liver iron measurement by MRI

For the measurement of liver T2* a single transaxial 10mm slice through the centre of the liver was acquired using a gradient echo sequence at eight different echo times (2.3 - 20.0ms). This was obtained during the same session as the cardiac T2* following the method described in Anderson et al (Anderson, *et al* 2001). This study had tissue validation for the liver iron assessments with an R=0.81 for all hepatic biopsies and an R of 0.91 P<0.0001 for the non-fibrotic liver biopsies. It is an accurate method

for calculating hepatic iron burden in no fibrotic liver samples although there is debate about the accuracy of results in the non fibrotic samples.

The estimated liver iron values obtained from the CMR were performed within 6 months of the blood samples. The serum ferritin, AST, haemoglobin, and NTBI were all measured on samples taken from the same venepuncture. The mean and the standard deviation were calculated and where required Student t tests were performed. All results are expressed as the mean (SEM).

5.4 Results: Aim 1: the relationship between hepcidin and LIC in thalassaemia

In Table 5.4.1.1 it can be seen that hepcidin mRNA was not significantly different in thalassaemia patients from control subjects despite the fact that LIC was between 6 and 8 fold increased above control liver tissue. This suggests that transfusional (n=18) or non transfusional (n=2) iron loading does not result in significant upregulation hepcidin synthesis in the liver in thalassaemia patients

Table 5.4.1.1: Hepcidin mRNA, ferritin, and LIC in patients.

Cohort	Hepcidin mRNA (% of mean) (SEM)	Ferritin (ng/ml) (SEM)	Liver iron (mg/g dw) (SEM)
Control N=10	100 (23.9)	30-400	0.35-1.36
All thalassaemia N=20	114 (14.6)	2448 (387.2)	8.1 (1.1)
Transfused patients N=18	110 (15.8)	2640 (405.6)	8.4 (1.2)
Non transfused TI N=2	123.4 (37.6)	719.4 (21.63)	5.7 (1.3)

Legend: Results are presented as the mean and (SEM). Liver hepcidin mRNA obtained from paraffin embedded sections are shown, in liver samples taken from healthy controls (n=10) in all iron thalassaemia patients (n=20), and in subgroups of 18 Transfused (14 TM and 4TI) and non transfused TI (n=2) patients. Values of hepcidin mRNA are normalized relative to the control liver samples. All controls had normal Perl's staining on liver biopsy and no evidence of siderosis, ferritins and liver iron quantification were not done and the range of ferritin and liver iron for normal adults is given. Patient ferritin values were the mean of the preceding 6 months and the liver iron result was that from the paraffin block used to perform the mRNA extraction.

5.4.1 Analysis of variables which may modulate hepcidin mRNA

As there appeared to be no difference in hepcidin mRNA in liver biopsies between thalassaemia patients, be they TI, TM or controls, an analysis of whether there was variability in hepcidin mRNA which could be accounted for by other factors such as

iron loading, liver function and hepatitis in thalassaemia patients taken as a whole (n=20).

The numbers are small however, and the results cannot truly be of any statistical significance, but will help to provide some generalisations. It can be seen that hepcidin mRNA is lower in patients with LIC values > 10mg/g dry wt than in those with values below this. Suggesting that iron overload may down regulated hepcidin. Liver hepcidin mRNA is higher in HCV RNA +ve patients (with active hepatitis) than those who are HCV RNA –ve suggesting that inflammation from hepatitis C results in increased hepcidin synthesis. AST may be increased from with iron overload or from hepatitis so for the analysis in Table 5.4.1.2 HCV RNA positive patients were excluded.

Table 5.4.1.2: Analysis of factors which may affect liver hepcidin mRNA levels

Variable (Units)	Category	n	Hepcidin mRNA (% of control)	sem	p value
Liver iron (mg/g dw)	>10	5	†85	12	0.3
	5-10	8	131	32	0.3
	<5	5	131	16	*0.05
Ferritin ¶ (ng/ml)	>3000	6	¶117	34	
	<1500	7	116	29	ns
	>60	4	^67.0	11.9	
AST ^ (iu/l)	<60	12	106.9	15.8	ns
	+	4	182	32.3	
HCV RNA	–	16	96.9	12.8	0.02

LEGEND: * Liver iron greater than 10mg/g/dw (n=5) had lower hepcidin mRNA and this was significant when compared to those with Liver iron below 5mg/g/dw (n=5) $p=0.05$.

$P=ns$ in patients with LIC>10 vrs<10 mg/g/dw

Patients who are HCV + ve have a significantly higher hepcidin mRNA than HCV-ve patients ($p=0.02$). Liver iron quantification was not available on 2 patients with TM who were excluded from this analysis.

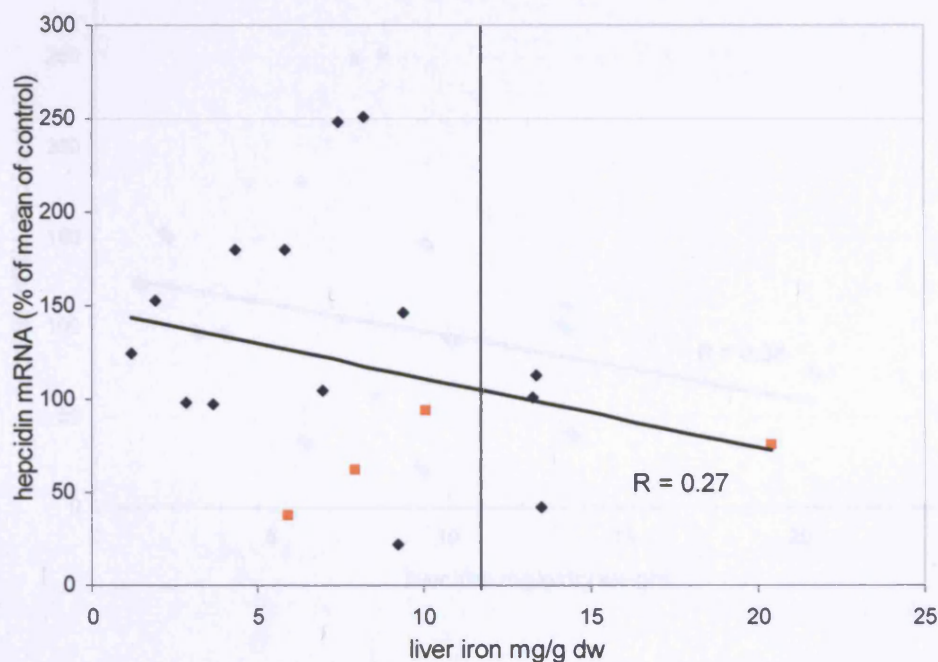
^AST excludes 4 patients who were HCV RNA positive.

¶ Ferritin >1501 and less than 2999 ng/ml (n=7) not shown on table values hepcidin mRNA% similar to levels in table.

5.4.2 Relationship of LIC to liver hepcidin mRNA

When LIC is plotted against hepcidin mRNA, it can be seen that there appears to be a weak negative trend between LIC and hepcidin mRNA in all thalassaemia patients, although this is not significant $P=0.27$.

Figure 5.4.2 a: LIC and hepcidin mRNA for all patients

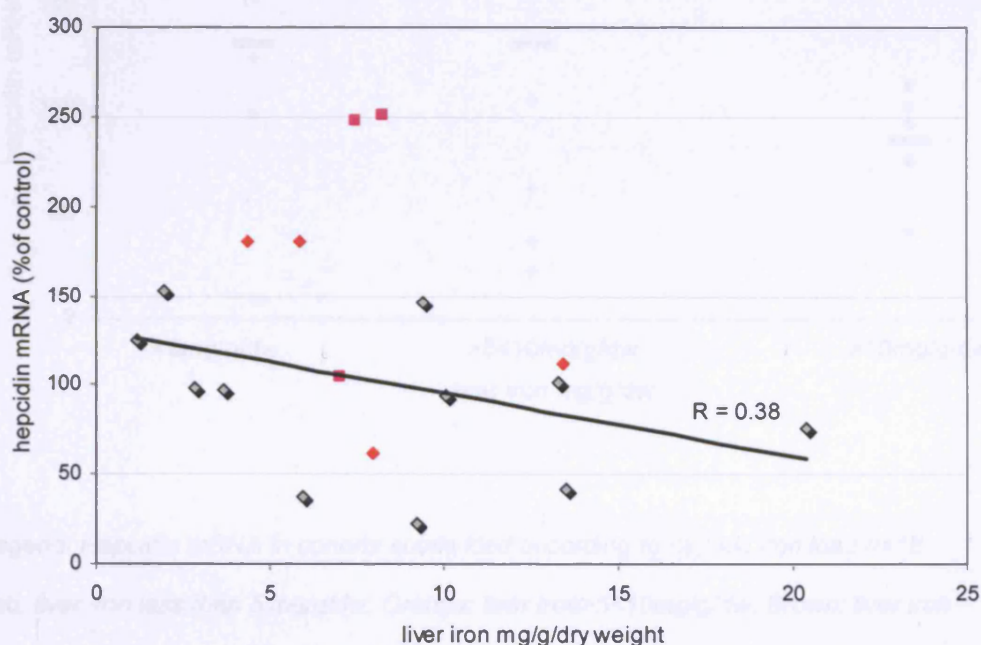


Legend; The relationship between liver hepcidin (expressed as % of that in control samples) and LIC is shown for all thalassaemia patients (n=18). It can be seen that for those patients with LIC values $\geq 10\text{mg/g dry wt}$ that hepcidin mRNA values are close to or less than control values. For LIC values $< 10\text{mg/g dry wt}$ with Hepcidin mRNA values show a large spread compared with controls ranging from 251% to 21%

Blue diamonds: all patients Orange squares: those with grade 3/4 fibrosis

As Table 5.4.1.2 suggested that HCV positivity was a confounding factor influencing hepcidin mRNA, an additional analysis of the relationship between LIC and hepcidin mRNA was undertaken where HCV +ve patients were excluded from the analysis. It can be seen in Figure 5.4.2.b that the negative trend between LIC and hepcidin mRNA is more pronounced when these patients (shown in pink) are excluded ($R=0.38$; $p=0.16$; $n=15$)

Figure 5.4.2.b: Liver iron and Hepcidin mRNA, excluding HCV RNA positive

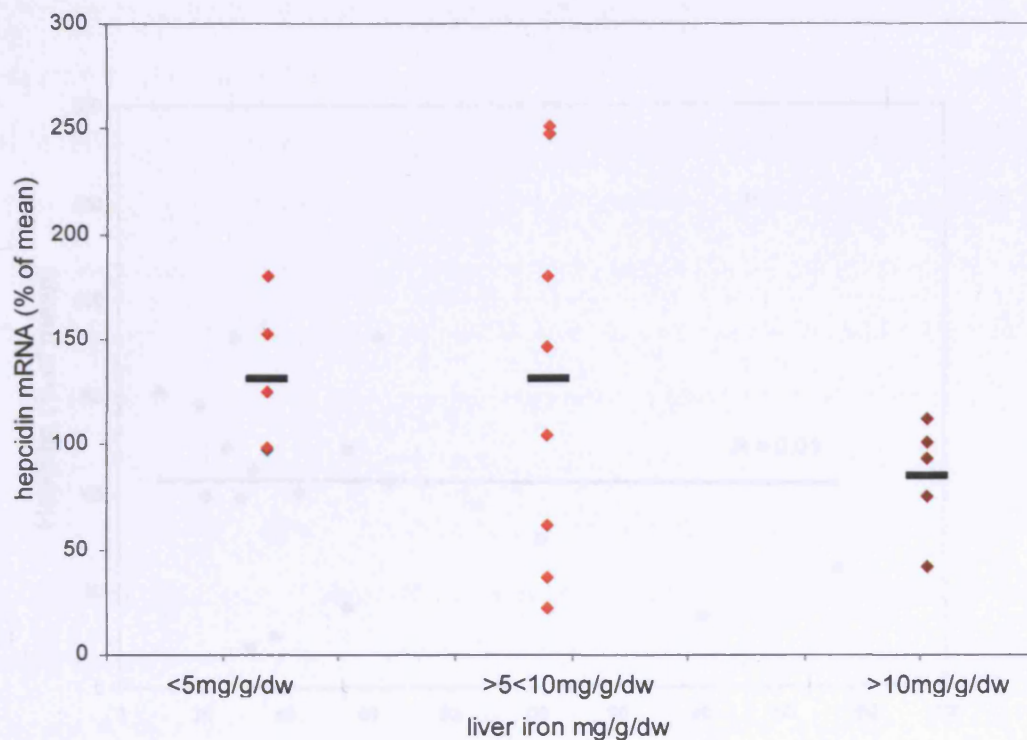


Legend: Black diamonds: liver iron in mg/g/dw compared to hepcidin mRNA (% of mean of control) in all patient excluding HCV RNA positive patients (n=15) excluding one patient with no liver iron measurement available) .

red squares TI patients.

Pink squares: HCV RNA positive patients (n=3) AST not available for one HCV RNA positive patient. Once those with inflammation have been excluded the liver iron does correlate with Hepcidin mRNA with higher iron burdens having lower hepcidin mRNA present.

Figure 5.4.2.c: Hepcidin mRNA subdivided according to LIC



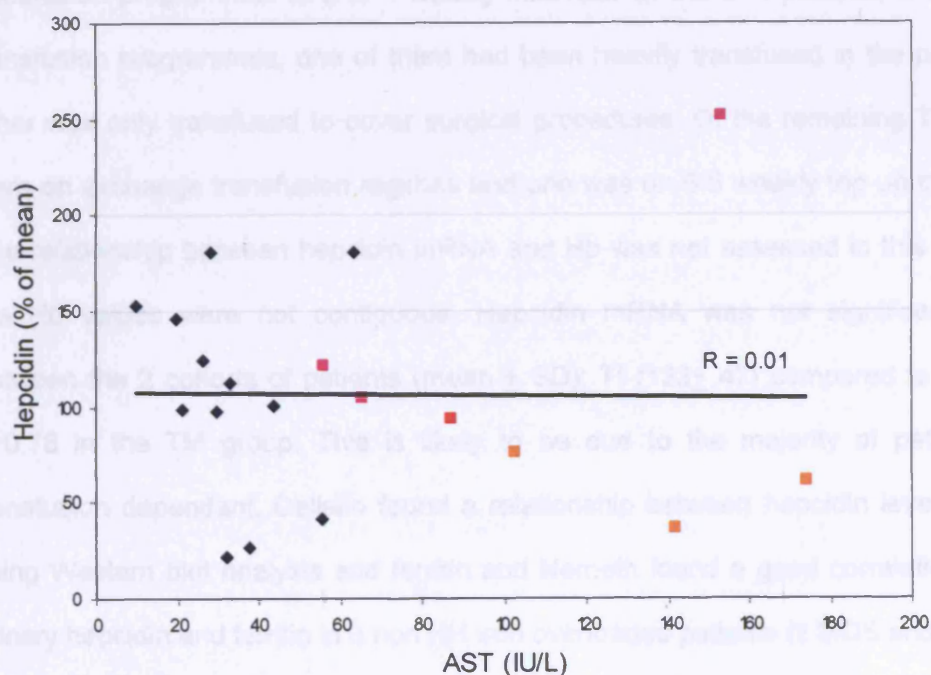
Legend: Hepcidin mRNA in cohorts subdivided according to hepatic iron load n=18

Red: liver iron less than 5 mg/g/dw; Orange: liver iron>5<10mg/g/dw; Brown: liver iron >10mg/g/dw. In cohort liver iron >10mg/g/d when compared to those with <5 mg/g/dw the hepcidin mRNA is significantly lower p=0.05. HCV RNA positive patients excluded.

5.4.3 Relationship of Hepcidin mRNA to serum AST

If HCV +ve patients are excluded (pink squares), there is a trend of decreasing hepcidin mRNA with increasing AST ($R=0.41$; $p=0.13$; $n=15$)

Figure 5.4.3.a: Relationship of Hepcidin mRNA to serum AST



Legend: The relationship between AST values and hepcidin mRNA is shown for 19 patients. AST not available for 1 patient who was HCV RNA positive. Blue diamonds HCV RNA negative thalassaemia patients; pink Square: those patients who were HCV RNA positive. Orange squares: those with greater than grade 3/4 fibrosis.

Some of the variability in AST can be accounted for the tendency for increased AST values with increasing LIC as demonstrated by the previously recognised positive trend between LIC and AST ($r=0.29$) when Hep C PCR +ve patients are excluded. Thus it appears that increasing liver dysfunction as shown by higher AST values are associated with a tendency for decreased hepcidin mRNA unless there is ongoing infection with hepatitis C which may lead to an increase in hepcidin mRNA.

5.4.4 Discussion: significance of hepcidin mRNA results

Both cohorts of patients were transfused. The TM patients were on regular transfusion programmes at 2 to 4 weekly intervals. Of the 6 TI patients, 2 were not on transfusion programmes, one of them had been heavily transfused in the past and the other was only transfused to cover surgical procedures. Of the remaining TI patients 3 were on exchange transfusion regimes and one was on 6-8 weekly top up transfusions. The relationship between hepcidin mRNA and Hb was not assessed in this analysis as the Hb values were not contiguous. Hepcidin mRNA was not significantly altered between the 2 cohorts of patients (mean \pm SD); TI (123 ± 47) compared to (110 ± 73) $p=0.78$ in the TM group. This is likely to be due to the majority of patients being transfusion dependant. Dallalio found a relationship between hepcidin levels in serum using Western blot analysis and ferritin and Nemeth found a good correlation between urinary hepcidin and ferritin in 3 non HH iron overloaded patients (2 MDS and 1HbSS).

More recently Aoki et al (Aoki, *et al* 2005) found hepcidin mRNA correlated well with ferritin in patients with HCV infection. We however did not see any relationship between the serum ferritin and liver hepcidin mRNA in patients with thalassaemia and iron overload ($R=0.21$; $p=0.98$; $n=18$). Those patients with a ferritin greater than 3000

ng/ml did not have a significant difference in the hepcidin mRNA expression (117%) ($p=0.99$) compared to those with a ferritin of less than 1500ng/ml (116%).

Hepatic iron burden is important in the expression of hepcidin and in keeping with the mouse study by Weizer et al (Weizer, *et al* 2004) we found a trend towards higher levels of liver iron burden having lower mean hepcidin mRNA values (85% vrs 131%, $p=0.05$). The reason for this paradoxical down regulation is unknown but one can hypothesise that the presence of NTBI at high level may have a role in this as shown in recent work by Nemeth on human hepatoma cells. Although NTBI may be an important factor in the down regulation of hepcidin it cannot be the only mechanism. Unfortunately we do not have contiguous sera samples from these patients to measure their NTBI. The patients with liver iron below 10 mg/g dry weight had a higher level of hepcidin mRNA expression compared to the controls as one would expect in response to increased iron load, this suggests that there is possibly a critical level of iron burden above which hepcidin expression becomes dysregulated and fails to increase with further increment in the iron burden and paradoxically its production goes down. It is possible that because hepcidin is synthesised by hepatocytes, once free iron in the form of the labile iron pool reaches a maximum threshold intracellular damage occurs and hepcidin production is reduced.

Transaminase levels are thought to be a reflection of liver damage and liver iron burden (Olsson, *et al* 1985). 4 patients with raised AST levels who were not HCV RNA positive had low mean hepcidin mRNA levels ($67\% \pm 12\%$), a higher mean liver iron ($11.09\text{mg/g/dw} \pm 24\%$) and increased levels of fibrosis (intermediate to high) compared to those with lower or normal AST levels ($n=12$) mean hepcidin mRNA ($106\% \pm 55$) mean liver iron 7.13 mg/g/ dw and only mild fibrosis; these numbers were too small to gain statistical significance but support the suggestion that significant liver damage (fibrosis score intermediate or greater) may lead to down regulation of hepcidin levels and

indeed this was recently noted Nemeth's group in patients who had had liver transplants or cirrhosis (Detivaud, *et al* 2005).

Inflammation results in up regulation of hepcidin as discussed earlier. Hepatitis C causes a chronic inflammatory mediated hepatitis. The predominant cell type involved in this inflammatory response is the T helper cell type 1 (Th1) (Neuman, *et al* 2001). Th1 cells release TNF α , IL2 and INF γ ; these cytokines have the ability to cause ongoing cellular immune response. TNF α stimulates macrophages in the liver to release of Transforming Growth Factor β (TGF β) which then goes on to stimulate the development of fibrosis. T helper cell type 2 (Th2) secrete IL4, IL6 and IL10 which in the presence of ongoing inflammation suppress the Th1 response and down regulate the TNF α and TGF β . There were 4 patients who were HCV RNA positive, all of whom had modestly elevated serum AST levels (mean 91.4 iu/l) and mild to high levels of fibrosis on biopsy were found to have raised hepcidin mRNA ($181\% \pm 79$) $p=0.01$. There was no difference in the HCV RNA positive and HCV RNA negative groups for ferritin (2701 ng/ml: 2350 ng/ml), AST (91.4 iu/l: 58.9 iu/l) or liver iron (7.5 4mg/g/dw versus 8.19 mg/g/dw) $p=ns$. In our patients who had chronic HCV carriage and variable degrees of fibrosis, it is possible that the Th2 response with IL4, IL6 and IL10 was the predominant factor in the inflammation and hence hepcidin was upregulated in the face of ongoing viraemia. The presence of excess iron burden in the presence of HCV infection results in significant worsening of liver fibrosis and hastens the onset of cirrhosis (Angelucci, *et al* 2000, Ardalan, *et al* 2004). It may be that in the presence of HCV infection, hepcidin mRNA expression increases more significantly in the presence of moderate iron loading and inflammation than it would if either was present alone.

It is however important to appreciate that in transfused thalassaemia patients iron overload is predominantly secondary to transfusion, is distributed differently and the role of fibrosis and inflammation are probably modified due to this. Transfusional siderosis is

initially reticuloendothelial and it is possible that a response may not be seen to the siderosis until moderate iron loading has occurred when the iron starts to enter hepatocytes thereby initiating stimulation of hepcidin synthesis. However as transfusional iron loading continues the intracellular labile iron pool continues to increase in amount and eventually cell death occurs due to hydroxyl free radical damage and hepcidin mRNA decreases in amount.

In conclusion it was noted in thalassaemia syndromes that hepcidin mRNA was down regulated at higher iron burdens, with greater levels of fibrosis and with higher AST levels. Hepcidin was upregulated with inflammation secondary to hepatitis C overriding the suppressive effect of iron and fibrosis on hepcidin mRNA expression.

It was decided to look at a wider population of patients and Kulaksiz et al had recently described a pro-hepcidin ELISA that identified a 10 kDa peptide which was down regulated in HH and in anaemia similar to that seen with other investigators who had used urinary hepcidin or hepcidin mRNA or serum hepcidin measurements using western blot analysis.

5.5 Results: Aim 2: pro-hepcidin, anaemia and iron overload in haemoglobinopathies

The relationship of serum pro-hepcidin to variables which might be predicted to influence levels such as iron loading, anaemia, transfusion and hepatitis were analysed, first in all patients, sickle and thalassaemia together and subsequently by individual patient group to determine whether the relationship of serum pro-hepcidin to these variables was different in different patients groups.

Several relevant differences between the designs of data acquisition occurred in the pro-hepcidin analysis compared with the liver mRNA for hepcidin. Firstly LIC was estimated from non-invasive measurements obtained during CMR as described in section 5.3.4. this method has been validated with liver biopsies. Secondly it was possible to include SCA patients in this analysis because LIC values were available and lack of liver biopsy tissue had excluded them from the first part of this study. Thirdly it was possible to obtain blood samples for analysis of serum pro-hepcidin in a systematic way in relation to blood transfusion. This allowed an analysis of the relationship between serum pro-hepcidin and anaemia, which was not possible with the liver biopsy analysis due to the retrospective nature of the analysis and the arbitrary timing of the liver biopsies with respect to transfusion. Finally it was possible to obtain blood samples for NTBI measurement on the same day as the serum pro-hepcidin samples, allowing an examination of the interrelationship of these variables.

5.5.1 Variables affecting prohepcidin, independent of diagnosis

In all patients grouped together, there a clear relationship between Hb values and serum pro-hepcidin with a significant difference in serum pro-hepcidin between patients with Hb values < 8 and those >10g/dl ($p < 0.0001$).

Table 5.5.1.1: Variables associated with prohepcidin levels in all patients

Variable (units)	Category	n	pro-hepcidin (ng/ml)	sem	p value
Hb (g/dl)	>10	19	128.78	7.11	<0.0001~
	8 -10	20	102.1	9.08	
	< 8	10	65.25	15.55	
# Liver iron (mg/gdw)	<5	27	110	8.26	ns
	5-10	11	94.73	12.88	
	>10	4	108	22.01	
Ferritin (µg/L)	<1500	29	101.9	8.87	ns
	1500-3000	7	118.9	9.6	
	>3000	12	114.5	9.8	
Ttransfused ¶	+	33	115	6.68	0.02
	-	16	84	10.9	
NTBI present	+	21	112	8.95	ns
	-	24	105	9.37	
ALT (iu/l)	>60	6	113	17.4	ns
	< 60	43	107	6.68	
HCV RNA ¢	+	4	55	18.6	0.01
	-	49	107	6.3	

Legend: * total of 49 patients were studied and n is the number of patients analyzed for the variable shown.

Liver iron was assessed by MRI (Anderson, et al 2001) in 41 patients

¶: Transfused are all patients currently requiring blood transfusion, including; all TM (>8 transfusions /y), currently transfused TI (TI: 1-8 transfusions per annum) and sickle cell patients who were on exchange transfusion programmes.

~ is the probability of Hb > 10g/dl compared to Hb <8 g/dl.(<8 vs. all patients >8g/d p =0.23)

∅: those patients who were HCV RNA positive and were excluded from analysis of Hb, NTBI, and liver iron, transfusion status and ALT. Those patients who had previously had HCV and were HCV RNA negative were included in the HCV RNA negative cohort

It is also apparent from Table 5.5.1.1 that there is also a tendency to lower serum pro-hepcidin in untransfused than transfused patients ($p=0.02$) the mean Hb at time of sample in transfused patients was 10.1g/dl and in the non-transfused (SCA and TI) was 8.5 g/dl and also a lower serum pro-hepcidin patients was found in HCV+ patients ($n=4$ $p=0.01$). A further finding in Table 5.5.1.1 is that serum pro-hepcidin appears to be lower in the 4 patients who are HCV RNA +ve than in uninfected patients. Surprisingly, this is the opposite of what was found for hepcidin mRNA in the liver biopsy samples where hepcidin mRNA appeared to be higher in the HCV RNA +ve patients however none of these patients were part of the hepcidin mRNA study.

Tables 5.5.1.1 and 5.5.1.2, show that the values for all patients on sub analysis were very similar to those when all 3 cohorts studied as a single group. There appears to be a significant relationship with transfusion status/anaemia. No relationship is seen with iron burden or serum ferritin.

Table 5.5.1.2: results (mean) for all patients according to type of anaemia

Diagnosis	n	Pro-hepcidin ng/ml (SEM)	Ferritin ng/ml (SEM)	Hb g/dl (SEM)	EPO mIU/ml (SEM)	Liver iron mg/g dw (SEM)	NTBI umol/l (SEM)
Control	10	119 (14.3)	50 (8.9)	13.2 (0.53)	14.7 (3.4)	ND*	0
SCA(all)	15	129 (9.6)	2304 (409)	9.3 (0.45)	68.6 (9.9)	4.75 (1.22)	0.21 (0.48)
SCA not currently transfused	3	138.7 (16.2)	1181 (562)	7.9 (0.64)	74.3 (18.9)	2.2 (1.27)	0.26 (1.14)
TM (all)	16	122 (8.3)	2824 (575)	10.8 (0.27)	50.6 (11.8)	4.65 (0.91)	1.48 (0.62)
TI (all)	18	78 (9.50)~	562 (193)	8.6 (0.33)	181.4 (24)	5.52 (0.83)	1.36 (0.56)
TI not currently transfused	15	78.5 (10.1)	461 (197)	8.5 (0.36)	114.2 (44.6)	4.9 (0.93)	0.8 (0.58)

Legend: Serum pro-hepcidin values, serum ferritin, LIC and Hb values are shown for control subjects and for patients with sickle and thalassaemia disorders. LIC was calculated from the relationship between liver T2 and liver LIC as described by Anderson.*

** Liver iron quantification not done in normal controls, assumed to be within the normal range 0.28 -0.44 mg/g/ dw (33 ± 7 ms)*

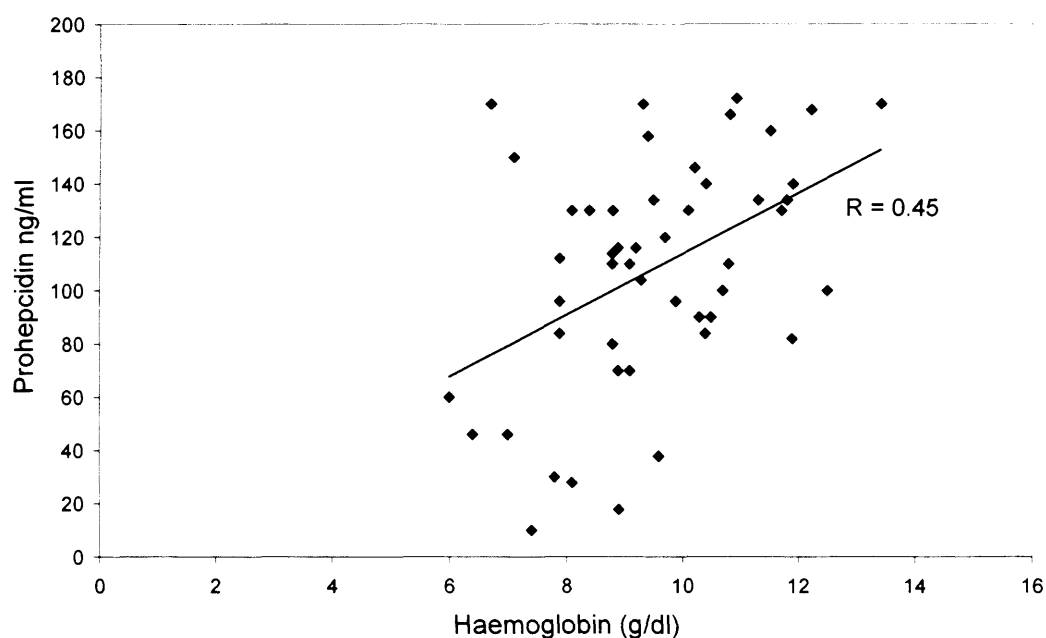
~ TI pro-hepcidin significantly lower than that of TM or SCA despite having similar iron burden.

There appeared to be a strong relationship with anaemia in subanalysis and this was confirmed on correlations.

5.5.2 Relationship between pro-hepcidin and anaemia

Further analysis of the relationship between Hb and serum pro-hepcidin in all patients, irrespective of diagnosis shows a correlation ($R=0.44$; $P=0.0012$; $n=49$). This is consistent with anaemia and hence hypoxia being a key factor in promoting pro-hepcidin values in plasma. This is also consistent with the known hypoxia mechanism for hepcidin synthesis and suggests that in this respect, serum hepcidin values behave in response to anaemia in a similar way to that previously reported for urinary hepcidin and liver mRNA (Detivaud, *et al* 2005).

Figure 5.5.2.a: Relationship of pro-hepcidin and haemoglobin in all patients



Legend: All patients analysed (n=49) excluding those with high CRP, HCV RNA positivity, renal impairment) as described in section 5.2.2

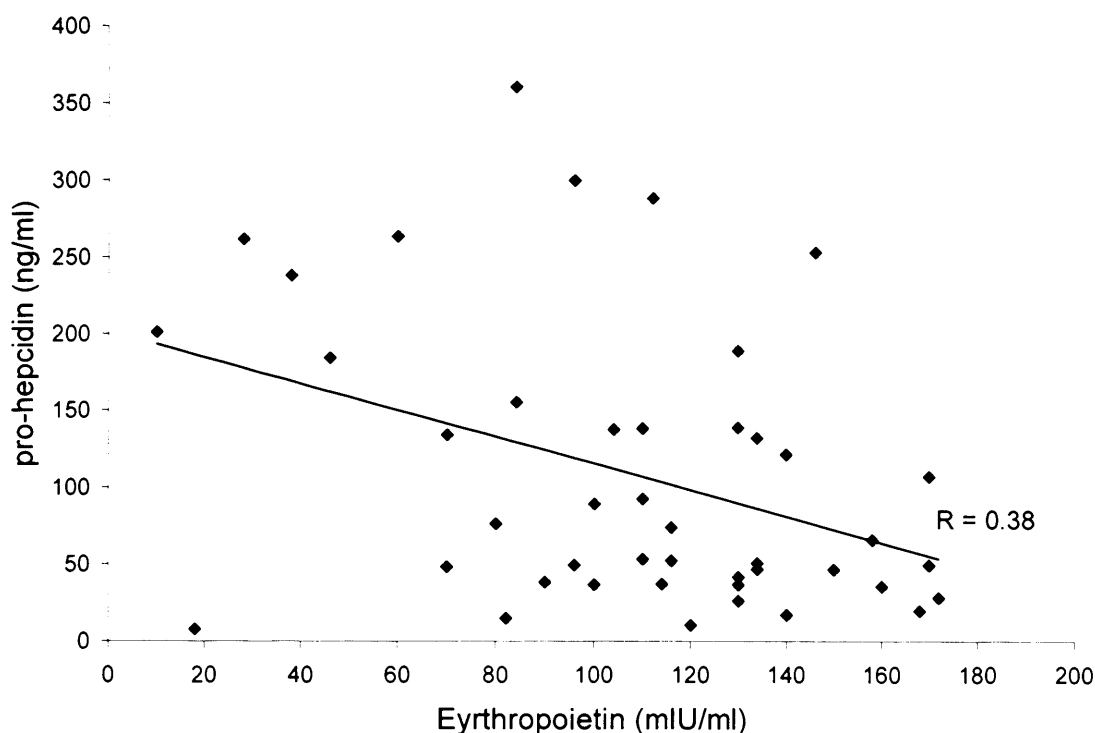
This figure shows a good correlation between Hb and pro-hepcidin ($R=0.45$; $P=0.0012$; $n=49$) however it was important to see if there were significant differences in

the 3 cohorts as they have different transfusion regimes and the degree of ineffective erythropoiesis is different in the groups.

When the three groups are assessed separately there is no significant correlation within individual cohorts, TM $R=0.38$ ($P=0.14$; $n=16$), TI $R=0.44$ ($P=0.067$, $n=18$) and SCA $R=0.25$ ($P=0.37$, $n=15$).

It is also possible that EPO may be playing an important role in this relationship with anaemia and pro-hepcidin and hence this was studied.

Figure 5.5.2.b: Relationship between EPO and pro-hepcidin



Legend: All patients analysed (n=42) excluding those with high CRP, HCV RNA positivity, renal impairment) as described in section 5.2.2

In the 3 groups when the data was analysed as a single cohort $R=0.38$; $P=0.01$; $n=42$. There was no correlation within individual groups; TM $R=0.33$ ($P=0.23$; $n=14$); TI $R=0.03$ ($P=0.9$; $n=17$) and SCA $R=0.22$ ($P=0.45$; $n=14$). From this figure it can be seen that a high EPO levels is associated with a low pro-hepcidin. If pro-hepcidin is the pro form of hepcidin found in the plasma this would explain how a high erythropoietic drive driven by EPO would cause suppression of pro-hepcidin/hepcidin and a resultant increase in the iron absorption in order to increase Hb synthesis. It is possible that the EPO driven hypoxia drive overrides the iron driven upregulation in the patients with thalassaemia.

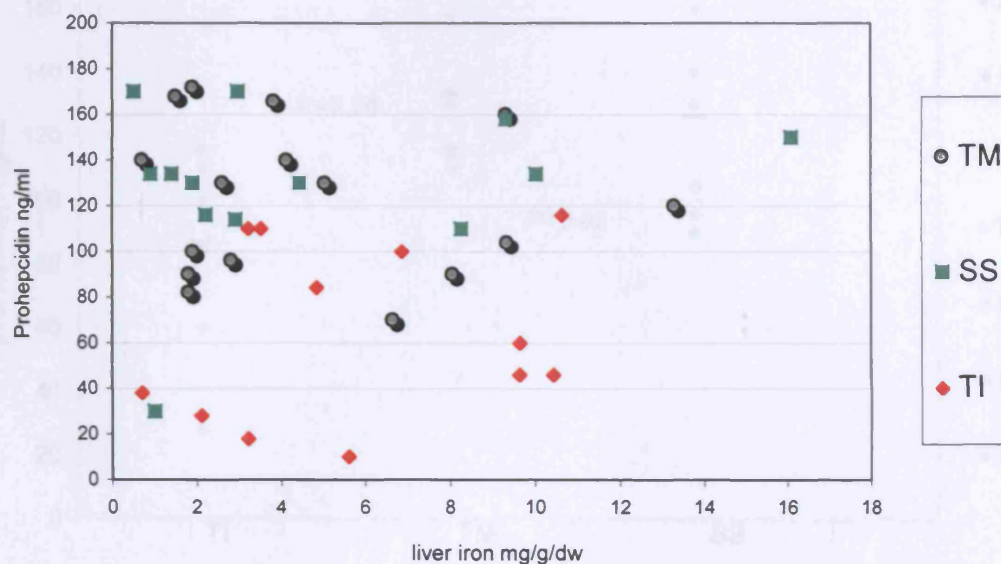
When all patients are grouped together in this analysis, there is no obvious relationship with serum prohepcidin and markers of iron metabolism such as serum ferritin and NTBI. This could be because differences between different classes of patients obscure trends within patient groups. Therefore in the subsequent sections, analysis of these data is undertaken by diagnosis.

5.5.3 Relationship between pro-hepcidin and LIC

There was no relationship between pro-hepcidin and LIC when all the patients were studied as a single cohort ($R=0.00$; $P=0.97$; $n=42$) nor when they were assessed as separate groups.

Figure 5.5.3.b: pro-hepcidin in 3 cohorts matched for LIC

Figure 5.5.3.a: Pro-hepcidin and LIC in patients

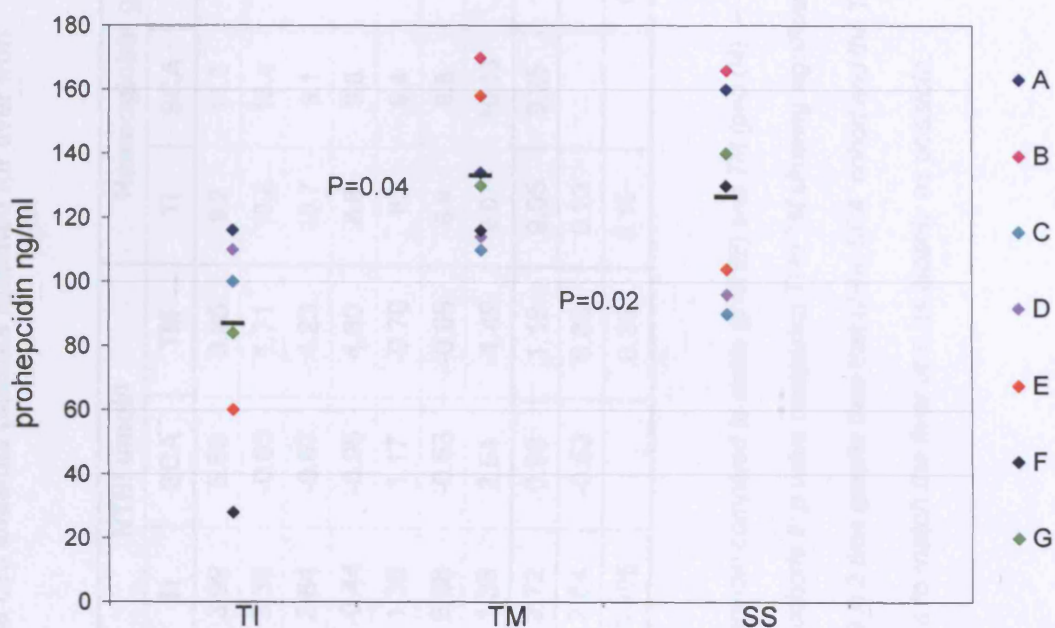


Legend: Black Squares: TM (n=16) red diamonds: TI: (n=13) and green squares: SCA (n=13)

All cohorts exclude patients as previously defined. Total patients n=42; 7 patients did not have CMR to assess liver iron 5TI and 2 SCA. There was no correlation in any of the groups of pro-hepcidin with iron burden. SCA $P=0.44$, TM $P=-0.59$, TI $P=0.60$

These results were unexpected, as other workers have shown a relationship with pro-hepcidin and liver iron and we had seen a relationship between hepcidin mRNA and liver iron as discussed earlier in this chapter. Figure 5.5.3a however showed that the pro-hepcidin was significantly lower at similar iron burdens in TI than either the TM or SCA groups. We decided to look at the results of patients matched for liver iron in the 3 cohorts to confirm this observation. Data from table 5.5.3.1 was used.

Figure 5.5.3.b: pro-hepcidin in 3 cohorts matched for LIC



Legend: patients matched for LIC as shown in table 5.5.3.1.

Patient characteristics

TI n=7 2 exchange transfused, 5 not transfusion dependant. P=0.02 comparing pro-hepcidin between TI and SCA

SCA n=7; 5 transfused 2 not requiring transfusions currently.

TM n=7 all transfusion dependant (p=0.04) comparing pro-hepcidin between TI and TM.

It is possible the degree of anaemia in TI patients may override the effect of iron burden on pro-hepcidin expression however, the sickle group also had a significant degree of anaemia and the mean Hb in SCA patients was 9.3 (± 1.76 g/dl) versus 8.6 (± 1.42 g/dl) in the TI cohort (p=0.24).

Table 5.5.3.1 Thalassaemia Intermedia, Major and sickle cell anaemia patients matched for liver iron

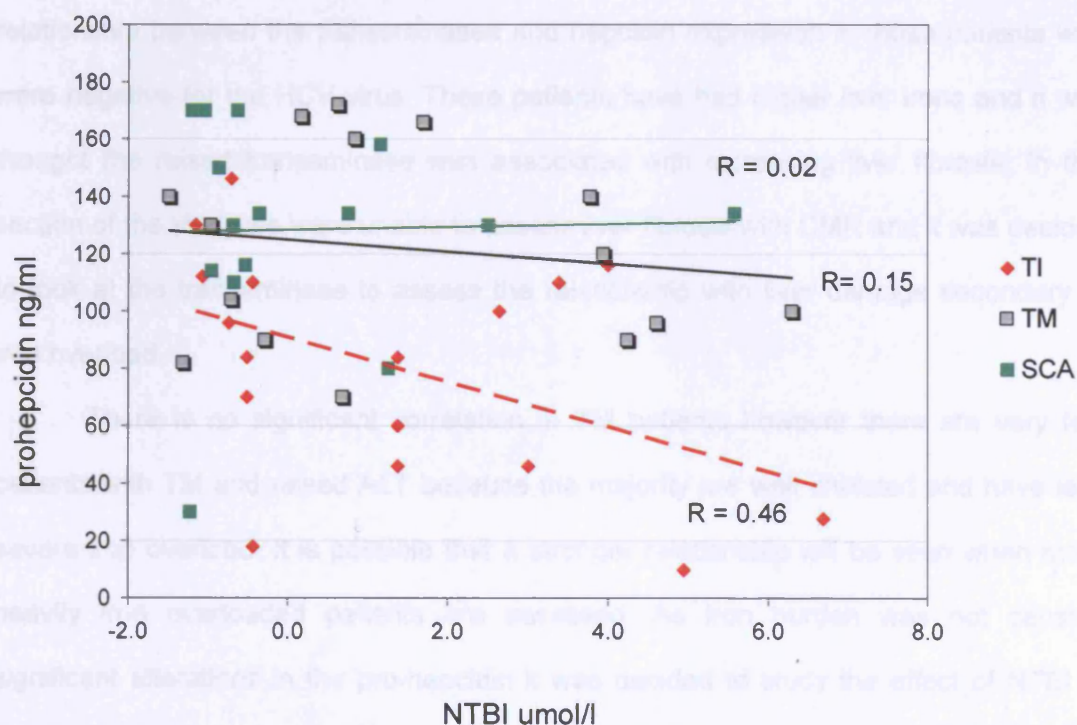
matched pair	liver iron mg/g dw			Prohepcidin ng/ml			NTBI umol/l			Haemoglobin g/dl		
	TI	SCA	TM	TI	SCA	TM	TI	SCA	TM	TI	SCA	TM
A	10.6	10.0	9.3	116	134	160	3.99	5.58	0.85	9.2	11.3	11.5
B	3.5	3.0	3.8	110	170	166	3.38	-0.63	1.71	10.8	13.4	10.8
C	6.8	8.2	8.0	100	110	90	2.64	-0.67	4.23	10.7	9.1	10.5
D	3.2	2.9	2.8	110	114	96	-0.44	-0.95	4.60	8.8	8.8	9.9
E	9.6	9.3	9.3	60	158	104	1.38	1.17	-0.70	6	9.4	9.3
F	2.1	2.2	2.6	28	116	130	6.68	-0.53	-0.96	8.9	8.8	11.7
G	4.8	4.4	4.1	84	130	140	1.38	2.51	-1.49	9.07	10.13	10.62
mean	5.80	5.71	5.70	86.86	133.14	126.57	2.72	0.93	1.18	9.05	9.25	10.65
median	4.80	4.40	4.10	100.00	130.00	130.00	2.64	-0.53	0.85	0.23		0.05
p value	0.74		0.76	0.02*		0.04*	0.19¶		0.35	0.16~		0.02#

*Legend: *The TI patients have a significantly lower serum pro-hepcidin compared to sickle (p=0.02) and TM (p=0.04). ~: The haemoglobin is not significantly different between the TI or SCA cohorts #: p value comparing TI to TM showing significant difference in the Hb.¶ although the difference in the NTBI is not significant it is 2 time greater then that of the SCA cohort and the TM cohort. These patients were not age or sex matched as the aim was to match the liver iron as closely as possible.*

5.5.4 Relationship between pro-hepcidin and NTBI

While figure 5.5.3.a suggests that liver iron stores are not an important regulator of serum pro-hepcidin, at least in the iron-overload range, in principle NTBI could modulate pro-hepcidin levels. Furthermore in Table 5.5.3.1, TI patients appear to have higher NTBI values than SCA patients despite having similar LIC values. An analysis of the relationship between NTBI and serum pro-hepcidin was therefore indicated. It can be seen in Figure 5.5.4a, that there is no relationship between pro-hepcidin and NTBI however in the TI cohort there is a trend towards falling pro-hepcidin levels when there is a high NTBI ($R=0.46$; $P=0.063$; $n=17$).

Figure 5.5.4.a: NTBI and serum pro-hepcidin in all patients



Legend: TI n=17; TM n=15; SCA n=15

There is a correlation between NTBI and pro-hepcidin in TI with those patients with the highest serum pro-hepcidin having the lowest NTBI ($R=0.46$). No correlation was seen with the TM and SCA patients and NTBI.

An analysis in the different patient groups figure 5.5.4a, show that the relationship of serum pro-hepcidin to NTBI appears to differ in the transfused SCA and TM patients compared to the TI. It is possible that the trend that is seen is related to the fact that TI patients have a raised EPO and are more anaemic than TM and SCA patients. In this situation the presence of NTBI secondary to ineffective erythropoiesis could be playing a role in down regulation of pro-hepcidin.

5.5.5 The relationship between pro-hepcidin and ALT

We had also shown in the study with hepcidin mRNA that there was a relationship between the transaminases and hepcidin expression in those patients who were negative for the HCV virus. These patients have had higher liver irons and it was thought the raised transaminase was associated with increasing liver fibrosis. In this section of the study we were unable to assess liver fibrosis with CMR and it was decided to look at the transaminase to assess the relationship with liver damage secondary to iron overload.

There is no significant correlation in TM patients however there are very few patients with TM and raised ALT because the majority are well chelated and have less severe iron overload. It is possible that a stronger relationship will be seen when more heavily iron overloaded patients are assessed. As iron burden was not causing significant alterations in the pro-hepcidin it was decided to study the effect of NTBI in these patients on pro-hepcidin expression to see if this may explain some of the differences seen.

5.5.6 Discussion: Factors influencing serum pro-hepcidin levels

As stated in Section 5.1, hepcidin synthesis is known to be positively regulated by inflammation and negatively regulated by hypoxia, anaemia and iron deficiency. An *in vitro* study has also suggested NTBI has a negative regulatory effect on hepcidin synthesis in cultured hepatocytes. In sickle and thalassaemia syndromes, the potentially competing effects of anaemia, iron overload, NTBI and inflammation on hepcidin synthesis and hence presumably serum pro-hepcidin are difficult to predict. The outcome of these competing effects could determine whether hepcidin is appropriately regulated in sickle and thalassaemia syndromes and might explain why thalassaemia syndromes are associated with hyper-absorption of dietary iron whereas sickle syndromes are typically not.

5.5.6.1 Pro-hepcidin and its relationship to anaemia

It was found that TI patients (n=18) had a significantly lower serum pro-hepcidin ($78 \pm 40.3\text{ng/ml}$) ($p<0.01$) compared to those with SCA (n=15) ($129 \pm 38.2\text{ ng/ml}$), TM (n=16) ($122.4 \pm 33.3\text{ng/ml}$) or control subjects (n=9) ($119 \pm 45\text{ng/ml}$) (table 5.5.1.2). Hb values were not significantly different between TI (8.6 g/dl) and SCA patients (9.3 g/dl) but were higher in the regularly transfused TM patients ($10.8\text{g/dl} \pm 1.1$). Patients with either SCA or TI who were not transfused had similar serum pro-hepcidin levels compared to transfused patients with the similar disorders (table 5.5.1.2). When all patients with Hb <8g/dl were compared to those with Hb > 10g/dl serum pro-hepcidin remained significantly lower ($p=0.001$) and this was most evident in the TI and TM cohorts with the SCA patients showing only a poor correlation between Hb and pro-hepcidin levels ($R=0.25$; $P=0.37$). This supports the role of anaemia as an important

modulator of serum pro-hepcidin. A high erythropoietic drive driven by EPO would cause suppression of pro-hepcidin/hepcidin and a resultant increase in the iron absorption in order to increase Hb synthesis. It is possible that the EPO driven hypoxia drive overrides the iron driven upregulation in the patients with TI. In support of this our SCA patients both transfused and untransfused had similar EPO levels to the TM cohort and were half those found in the TI group.

In TI the continued suppression of pro-hepcidin due to anaemia would lead to continued iron absorption via the gastrointestinal tract and patients would develop iron overload. In SCA the pro-hepcidin does not appear to be down regulated in response to anaemia and hence iron overload does not develop secondary to increased GI absorption. This is similar to the findings by Ezech et al (Ezech, *et al* 2005) where the expression of pro-hepcidin in SCA patients was found to be similar to that of normal controls despite the anaemia.

The reasons for this difference between the 2 different anaemia disorders may be the degree of ineffective erythropoiesis. TI is a disorder of ineffective erythropoiesis and SCA is a haemolytic anaemia.

5.5.6.2 *Pro-hepcidin and its relationship to Iron*

No difference was seen between the three cohorts according to the iron burdens. This may be because there were too few patients with liver irons > 10 mg/g/dw (table 5.4.1.1.) It was shown by Nemeth that NTBI down regulated hepcidin but we did not find any correlation between pro-hepcidin and NTBI although there was a trend towards suppression of pro-hepcidin levels at higher NTBI levels in TI figure 5.5.4a although this is not a significant correlation. The difference in serum pro-hepcidin between TM, TI and SCA persisted when the 3 groups were matched for liver iron ($p < 0.001$) (table 5.5.3.1.).

NTBI was lower in SCA (mean $0.93 \pm 2.41 \mu\text{mol/l}$; median $-0.53 \mu\text{mol/l}$) compared to TM ($1.18 \pm 2.47 \mu\text{mol/l}$ median $0.85 \mu\text{mol/l}$) and 2 fold lower than in TI ($2.72 \pm 2.28 \mu\text{mol/l}$; median $2.64 \mu\text{mol/l}$) when patients were matched for liver iron.

It is possible that NTBI, present early in TI secondary to high ineffective erythropoiesis, may lead to a similar paradoxical decrease in hepcidin synthesis reported in model systems and that this is reflected by reduced serum pro-hepcidin. Although levels of NTBI and liver iron did not differ significantly in TI and TM, transfusion and chelation therapy in TM may override NTBI mediated down regulation of pro-hepcidin.

There are several possible factors which need to be considered with regard why NTBI may be low in SCA and how this may affect iron loading and pro-hepcidin. The first may relate to the effects of inflammation on serum pro-hepcidin. SCA patients exist in some respects to 'a chronic inflammatory state' due to the nature of microvascular occlusion seen in this condition. Indeed it is known that several cytokines including IL-6 are increased in SCA patients, even in steady state IL6 and IL1 in sickle (Bourantas, *et al* 1998, Taylor, *et al* 1997). Since IL6 has a central role in hepcidin metabolism, it is possible that increased hepcidin synthesis consequent to high IL6 secretion decreases iron absorption from the gut and iron release from macrophages. Decreased iron release from macrophages may in turn account for the lower NTBI seen in SCA patients compared with LIC matched TI or TM patients (Table 5.5.3.1)

It is not immediately apparent why NTBI levels should be lower in SCA patients than TI at matched iron levels. However, one difference between SCA and TI patients is that in SCA results predominantly from haemolysis whereas in TI, ineffective erythropoiesis. Ineffective erythropoiesis may lead to a greater chance of NTBI production due to the relatively hypoxic environment in the bone marrow leading to slower binding of iron to transferrin.

Thus the results in this section show that anaemic patients with TI have lower pro-hepcidin than in SCA patients with similar levels of anaemia and iron loading. Furthermore TI patients have higher NTBI levels than SCA at matched levels of iron loading. It is hypothesised that the uptake of NTBI delivered to the hepatocyte may be a key factor in determining lower hepcidin synthesis and hence higher iron absorption in TI than SCA.

Fibrosis and ultimately hepatic cirrhosis occurs in thalassaemia patients secondary to iron overload. This results in a rise in the transaminases and again unlike with the hepcidin mRNA cohort we did not see any correlation with ALT and pro-hepcidin. The small number of patients with a significant transaminitis may again account for this.

5.6 Conclusion

In conclusion pro-hepcidin is clearly down regulated in response to anaemia and raised EPO. Pro-hepcidin shows a downward trend with NTBI in TI although there is no relationship seen with TM or SCA. There was no clear alteration of pro-hepcidin levels in the presence of increasing liver iron burden neither (ferritin nor liver iron) however this may be due to the small number of patients with a high liver iron. Kulaksiz et al also found no significant relationship between pro-hepcidin and ferritin or transferrin saturation (Kulaksiz, *et al* 2004).

Hepcidin mRNA was found to have a downward trend with increasing LIC or transaminitis reflecting an increasing iron load. There was no relationship found with the ferritin and hepcidin expression.

Pro-hepcidin measurements using the ELISA need to be interpreted with caution. It is important to ensure that the assay is performed at a similar temperature if the results between two test series are to be analysed together. It is also important to ensure that the results fall in the 'linear' portion of the standard curve i.e. between 4-400ng/ml. We attempted to overcome these issues by performing the assay on two days when the room temperature was similar i.e. between 20-22 °C. We also ran the same control samples on each day these showed some variation. It is important to note that in our patients most of the results for individual disorders were fairly similar within a group as reflected by the low SEM results as seen in table 5.5.1.2.

Further work needs to be undertaken to see if the relationship between NTBI and pro-hepcidin is real or if this is a reflection of the anaemia. We plan to perform hepcidin measurement on the serum samples once this assay is developed

Chapter 6

Discussion and Conclusions

The purpose of the work presented in this thesis was to examine the relationship between NTBI and iron overload in patients with haemoglobinopathies. Although NTBI has historically always been associated with iron overload, we have shown that NTBI is present in young children at high levels before they have developed significant iron overload. It is probable that this NTBI arises as a consequence ineffective erythropoiesis and indeed in the small number of patients examined we saw a fall in the level of NTBI post transfusion in previously untransfused children.

It has also been shown in chapter 4 that higher hepatic iron burdens will result in a slower rate of improvement of cardiac iron loading. In addition SCA patients rarely develop cardiac iron loading. This may be to RE sequestering of toxic iron species and indeed NTBI is present at much lower levels in patients with SCA compared to those with TM even when matched for liver iron. The role of hepcidin may be important in regulation of iron in haemoglobinopathies and it has been shown that in patients with TI, pro-hepcidin levels are significantly lower than in patients with SCA or TM at matched levels of hepatic iron.

The results in the individual chapters will be summarised below along with a discussion on limitations and clinical application if any.

6.1 The relationship between markers of ineffective erythropoiesis, oxidative damage and NTBI in children with thalassaemia

6.1.1 Important observations:

The presence of NTBI early in childhood in thalassaemia patients is an important observation and has not been described previously. It is likely, based on the analysis of data available, that ineffective erythropoiesis is an important contributing factor to NTBI formation in patients who have accumulated only small quantities of excess storage iron.

It was also observed that in thalassaemic children sTfR and erythropoietin correlated well with the degree of the anaemia. There appeared to be very little oxidative damage as measured by levels of MDA and protein carbonyls in these children who were on the whole well transfused and chelated. There was also no relationship between markers of ineffective erythropoiesis and markers of oxidative damage. In addition neither of these factors had any correlation with the presence or absence of NTBI.

There does not appear to be any relationship between markers of oxidative damage and iron burden in well-chelated children. This is probably due to the duration of exposure to toxic forms of iron being less than in the adult patients who had less chelation therapy in childhood. Since the examination of this thesis Walter et al (Walter, *et al* 2006) published data showing that MDA levels were raised in SCA and TM and this correlated to liver iron burden. It is important to note that the study in this thesis is a longitudinal study and once data collection is complete, then the longitudinal analysis will provide more accurate information in individual patients on the relationship between NTBI and markers of oxidative damage.

6.1.2 Limitations and need for further studies

- *Sample collection and handling*

This as discussed in chapter 3 has been a concern. Any defrosted samples have been discarded and only those that have arrived in a frozen state are used. Samples are sent in 0.25ml aliquots and hence only one aliquot is defrosted for analysis, samples are not repeated defrosted and frozen. All the markers of oxidative damage have been analysed wherever possible on samples that have not previously been defrosted although in the case of measurements of EPO and sTfR we have often had to use previously defrosted samples.

- *Lack of normal range of MDA and carbonyls in children*

This has been a significant concern and we have not been able to obtain healthy control samples from children for MDA and carbonyl levels. This is due to the difficulty in obtaining ethical approval for sample collection in healthy children. Attempts are ongoing to address this issue.

- *Need for more children re and post first transfusion samples*

The data in section 3.8.3 needs to be further validated. Currently there are too few numbers of children having their first transfusion although since this analysis has occurred, newly diagnosed children with TM have presented both at NTCK, and HSC. It is hoped that data from these children will corroborate the preliminary findings.

- *Cross-sectional analysis*

This is a cross-sectional analysis and once more longitudinal data are available, it may be possible to ascertain in more detail if there is a relationship between markers of oxidative damage and NTBI/ iron overload.

6.1.3 Clinical implications

There are no real studies on the natural history of iron overload in thalassaemic children who are chelated looking at the impact of chelation therapy on markers of

oxidative damage. This study may potentially have significant impact on options for treatment and chelation as it is assessing the effect of current standard treatment in UCLH/HSC children as well as those who initially were receiving less rigorous treatment in NTCK. It will be important to continue with the longitudinal follow-up that is part of this study to see if oxidative damage increases with age and prolonged exposure or if a patient becomes non adherent to therapy and develops significant iron burden.

6.2 Relationship of hepatic iron and NTBI to myocardial iron and LV function: contrasts between SCA and Thalassaemia disorders

Iron deposition occurs progressively but unevenly between different tissues as continued transfusion support is provided to children and patients develop endocrinopathies and cardiac failure if inadequately chelated. The relationship between NTBI and iron loading particularly in the liver and myocardium was assessed in adult patients with thalassaemia syndromes and SCA. CMR is a valuable tool in the management of iron overload and should be offered to all patients on regular transfusion regimes. It has provided a great deal of information on the distribution of iron in the body; this has lead to improved management of patients. Although tissue validation of cardiac T2* as a methodology is still awaited, it nevertheless assesses a clinically important parameter and has likely prognostic significance.

6.2.1 Important observations

In patients with thalassaemia syndromes we found a relationship between cardiac T2*, cardiac R2* and cardiac function, as measured by LVEF, consistent with previous observations by others. The role of longitudinal monitoring is however previously unreported and vital to the interpretation of these measures. We conclude

from a longitudinal analysis that a liver iron >10 mg/g/dw was associated with a significantly increased risk of the cardiac T2* worsening. In addition a liver iron less than 10mg/g/dw was required prior to seeing an improved in the cardiac T2*.

Ferrioxamine is produced when DFO binds to iron in a 1:1 ratio; if formed within cells, it exits only slowly, over 1-2 days. Because, ferrioxamine has been previously used as a contrast agent for MRI imaging, we had concerns that ferrioxamine might alter the cardiac T2* signal. This issue was addressed using sequential monitoring of T2* in one patient. The degree of change in the T2* over a period of 48 to 84 hours is important and it is probably best to advice patients to have the CMR assessment at the same point in their chelation cycle on each occasion i.e. if they normally have the CMR assessment whilst they have their DFO infusion, then future CMR assessments should also be undertaken with the DFO infusion being administered. This would remove any variability due to slow ferrioxamine clearance from the cells.

It has been suggested that damage to the myocardium may be mediated by NTBI (Glickstein, *et al* 2005), through generation of free radicals (Rachmilewitz, *et al* 2005). Whether this is a direct effect or not, NTBI is known to be cleared by myocardial cells, leading to increased iron deposition within the myocardium and ultimately to increased storage iron as well as increased intracellular labile iron that can be directly damaging to myocardial cells. We have found that patients with SCA tend to have much lower levels of NTBI at matched levels of iron burden compared to TM patients. The NTBI present in SCA and in TM adult patients will predominantly be due to excess total body iron, secondary to transfusion, rather than due to ineffective erythropoiesis. It has been shown in SCA that, even at steady state, there are increased inflammatory markers and cytokines such as IL1, IL6, IL8 and TNF (Walter, *et al* 2006, Makis, *et al* 2000b). These cytokines may down regulate the levels of NTBI at matched levels of iron loading by causing sequestering of iron in the RE system.

The TI cohort are transfused less frequently than TM patients but they are more prone to develop iron loading compared to the SCA patients because of the role of increased gastrointestinal iron absorption.

6.2.2 Limitations and need for further studies.

- *MRI methodology and use of the Cardiac T2**

There has been significant debate on the use of this method in the medical literature and whether the cardiac T2* measures myocardial iron. This is because there is no cardiac tissue validation of the T2* and liver tissue validation was used to calibrate the liver T2*. Other workers have carried out successful calibrations using endomyocardial biopsy and T2 (Mavrogeni, *et al* 2005). In section 4.6.1, we have confirmed previous observations that there is a relationship between cardiac T2* and LVEF. This implies that despite the lack of tissue validation, the cardiac T2* is measuring a clinically relevant parameter with prognostic value. Studies are currently underway at the Royal Brompton Hospital CMR unit to validate this methodology using endomyocardial biopsies and cadaveric hearts from thalassaemia patients that have been donated to the unit.

- *The use of liver iron for matching patients*

We have used hepatic iron load to match our patients working on the assumption the hepatic iron is a reflection of total body iron (Angelucci, *et al* 2000). However the cumulative iron load is very different in the two groups of patients as the SCA cohort have received exchange transfusions predominantly. It is possible that cardiac iron loading is seen in SCA when cumulative iron load or the years of exposure to iron is examined. There is a clear need for a long term study to assess this.

- *Ferrioxamine and its effect on T2**

We have only studied one patient. This work needs to be carried out in a larger group of patients prior to concrete conclusions being made.

- *Speciation studies on NTBI*

Our studies focussed on the measurement of 'total NTBI' using the classic NTA assay. However the possibility the proportions of NTBI species differ between SCA and other anaemias needs to be explored. In particular it will be of value to examine the relationship between total NTBI and labile plasma iron (LPI) a subspecies of NTBI that is redox active. It is not clear whether LPI is cleared by tissues in a similar way to other NTBI species and this could be important in how iron is distributed in SCA compared with thalassaemia syndromes.

6.2.3 Clinical implications

MRI assessment of iron loading is a valuable and clinically relevant technique. It should be offered to all patients on transfusion programmes. The cardiac T2* when assessed in conjunction with the LVEF is clinically relevant and can be used to change the chelation regime given to a patient, with intensification of chelation at low cardiac T2* i.e. <10 ms and a falling LVEF.

In addition, in patients where there is already cardiac iron loading, it is important to reduce the hepatic iron burden below 10mg/g/dw before any significant improvement is seen in the cardiac T2*. It is important to ask patients to have their CMR assessment in a standardised chelation routine, i.e. if the assessment is performed off all chelation for 48 hours then all subsequent scans should occur at a similar time point. Further work is needed on the effect of ferrioxamine.

Even though we did not see this in our patients, SCA patients may develop cardiac iron loading. Until we have more longitudinal data it will still be important to

monitor for this complication in SCA. The analysis in this thesis of the relationship between liver and heart iron was observational rather than pre-planned. Further longitudinal studies are clearly needed to examine the relationship between body iron and heart iron, particularly in SCA patients.

6.3 Hepcidin metabolism, iron overload and NTBI in thalassaemia and sickle disorders

6.3.1 Important observations

The recent discovery of iron regulatory proteins such hepcidin have provided great insight into how gastrointestinal iron absorption and iron release from macrophages is modulated. The relationship between hepcidin expression and iron burden in liver biopsies in thalassaemia syndromes was examined and we found that Hepcidin mRNA showed a downward trend in relation to an increasing liver iron burden or transaminitis reflecting an increasing iron load. There was no relationship found with the ferritin and hepcidin expression. The numbers are very small and do not reach statistical significance.

The relationship between pro-hepcidin and anaemia and iron overload was assessed in sickle and thalassaemia syndromes. Pro-hepcidin is clearly down regulated in response to anaemia in thalassaemia syndromes but less obviously in SCA. This relationship was also seen with high EPO levels associated with a lower pro-hepcidin. No relationship was seen between pro-hepcidin and liver iron or serum ferritin. There was a significantly lower pro-hepcidin in TI patients compared to those with SCA or TM then matched for liver iron, implying that the down regulatory affect of anaemia overrides the upregulatory effect of iron overload. In addition NTBI was having a suppressive effective on pro-hepcidin in TI. Recent publications, since this thesis was examined by

Kattamis (Kattamis, *et al* 2006) and Gardenghi (Gardenghi, *et al* 2007), have both suggested that ineffective erythropoiesis is a key driver of hepcidin mediated iron absorption, similar to our findings.

6.3.2 Limitations and need for further studies.

- **Measurement of Hepcidin mRNA**

The study was retrospective and further work should be performed prospectively to confirm our findings and allow us to perform a range of measures simultaneously to the timing of the biopsy. Recently Jenkins *et al* (Jenkins, *et al* 2007) found increased hepcidin mRNA levels in liver biopsies of SCA and TM patients. Ideally future studies should be undertaken prospectively with simultaneous measurements on NTBI, Hb and plasma hepcidin at the time of biopsy. This would allow a closer analysis of factors that modulate hepcidin synthesis in the liver and its release into plasma. It will be useful to examine to what extent anaemia or hypoxia override the effects of iron status. A further factor that needs to be controlled for is the short-term effect (if any) of chelation administration on hepcidin synthesis.

- *Pro-hepcidin assay and its relationship to hepcidin.*

This has been discussed in section 5.1.6. It is important to ensure that the assay is performed in similar conditions. Studies are clearly needed to examine the relationship between plasma pro-hepcidin and plasma hepcidin. Hitherto this has not been possible because of technical issues surrounding the measurement of hepcidin in plasma. However since the initial submission of this thesis, a plasma assay for hepcidin has been developed. Matched plasma aliquots have been retained on SCA and thalassaemia patients measured in this study and analyses are underway to

examine how plasma and hepcidin and pro-hepcidin levels relate to each other in these patients.

- *Need for longitudinal studies to include patients with high iron burdens*

None of our patients had very high LICs so the effect of high hepatic iron loading on pro-hepcidin were not examined. Future studies should also ideally include longitudinal analysis of the effects of progressive iron loading on (pro) hepcidin and NTBI.

- *Need for study of relationship of inflammatory markers, hepcidin and NTBI in SCA*

Our observations are consistent with the notion that NTBI levels are depressed in SCA relative to body total iron levels compared with thalassaemia major patients and that elevation in (pro) hepcidin may be a mechanism for this effect. Prospective studies, designed specifically to examine the relationship between inflammatory markers, plasma hepcidin and NTBI levels are indicated in SCA. The question of whether a chronic inflammatory state is the key factor responsible for depressed levels of NTBI in SCA will require clarification. This may involve the study of the relationship between inflammatory markers plasma hepcidin and NTBI levels in steady state and during vaso-occlusive episodes.

6.3.3 Clinical implications

Pro-hepcidin appears to be suppressed in our patients with TI and this appears to be related to the degree and severity of the anaemia. This results in increased gastrointestinal iron absorption. It appears that the effective of anaemia overrides the effect of iron overload in TI. It is possible that a hepcidin analogue that is orally active may be developed in the future and this would at high levels suppress iron absorption in these patients. this could have great implications for the management of iron overload in TI. A further possibility is that raised levels of hepcidin in TM could prevent the

secondary redistribution of iron from macrophages via NTBI to heart, pituitary and other tissues. In principle this could be achieved by giving exogenous hepcidin analogue or, less appealingly, by the induction of an artificial inflammatory state.

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Appendix 1

Children from UCLH/HSC

patient	cumulative iron (grams)	cumulative DFO	NTBPI	Serum transferrin rcptr	Serum EPO	carbonyls	MDA
1	2.37	19.5	3.7	23.08	1841.05	0.67	0.200
	2.64	41.1		13.64	44.57944	0.75	0.230
	2.90	70.0	4.5	0.05		0.73	0.140
	3.20	105.4			26.32		-0.126
	3.20	115.0	-0.8	33	31.27		-0.099
	4.30	189.0			900.62		-0.070
2	1.01	0.0	3.07		53.71	0.96	0.0.
	1.77	0.0	4.3		173.9	1.43	0.200
	2.59	0.0	10.4		177.8475		0.003
	3.65	28.5	0.6		98.18644		0.001
	4.53	91.5	8.1				
	5.17	145.5			90.98305		-0.057
3	9.50	73.9	4.2	3.81	27.89	0.67	0.290
	10.20	127.9	3.3	6.54	77.75701	0.62	0.170
	11.35	211.4	3.2		41.98		-0.042
	12.55	295.0	6.5		21.36		0.045
	12.79	313.0	5.2		56.74576		-0.019
	13.57	354.5	4.3				
	15.13	435.0		14.6	63.18644		0.042
4	3.08	0.0	4.3	7.88	400.26	0.82	0.160
	3.70	0.0	4	15.24	403.24	0.65	0.360
	4.57	40.0		7.64	346	0.73	0.330
	5.49	88.7	0.24	6.22		1.03	0.240
	6.41	150.4	5.7		195.3051		0.042
	6.69	164.7	2.8		192.678		0.036
	7.94	253.7	8.4				
	8.98	328.7			74.54237		-0.006
5	0.24	0.0	6.64		289.2034		-0.108
	0.39	0.0	4.4				
	0.77	0.0			98.69492		-0.041
6	0.00	0.0	-0.26	7.67	3276	0.32	0.090

	0.09	0.0	5.65	19.4	5215	0.82	0.160
	1.23	0.0	1.77	4.68		0.81	-0.030
	2.12	0.0	0.5	4.13	213.4	0.51	0.050
	2.80	0.0	1.7	6.91	221.4	0.69	0.060
	3.60	0.0	-1.5	5.87	207.03	0.69	-0.100
	4.76	0.0	5		107.2		-0.088
	5.01	21.0	3.9		146.85		-0.059
	5.26	34.5	3.85				
7	2.17	0.0	6.8	6.4	225.52		insuff
	2.82	0.0	5.3	8	192.54	0.70	0.030
	3.71	32.6	7.49	3.58	106.41	0.37	0.150
	4.91	99.9	5.02	2.47	24.95327	0.70	0.010
	5.70	151.9	5.71	1.68	32.76	0.62	0.090
	6.79	191.9		2.09	76.82243	0.77	0.100
	8.81	341.9	9.7		70.38983		-0.085
	9.11	365.9	12.1		52.16949		-0.171
	10.36	467.9	4.9				
	10.69	497.9	6.8				
	11.68	588.9			55.13559		-0.087
8	2.21	0.0	5.3	3.74	197.63	0.18	0.110
	2.75	7.5	8.52	4.74	212.06	0.71	0.090
	3.46	57.5	5.61	4.1	209.07	0.58	0.164
	4.00	101.5	78.82	7.03	229.43	0.84	0.080
	4.74	167.2	5.6	3.32	15.4	1.14	0.182
	5.53	233.5	5.6	4.1	88.27119	0.82	0.080
	6.29	301.5	5.1				
	6.66	335.8	7		145.3051		-0.101
	7.44	391.8	5				
	7.66	403.8	3.6				
	8.48	467.8			81.23729		-0.087
9	0.08	0.0	0.3		74.0339		-0.124
	0.17	0.0	3.7		38.18644		-0.101
	0.35	0.0	-0.34		68.01695		-0.083
	0.44	0.0	-1.5				
	0.53	0.0	1				
10	0.99	0.0	-0.5			0.38	in suff
	1.23	0.0	5.51	3.9	120.72	1.29	in suff
	1.88	4.4	6.1				
	2.14	11.9	6			0.42	0.330
	2.70	36.0	5.2	4.15		0.62	in suff
	3.32	70.9	-0.33	4.15	49.62	0.61	0.040
	5.10	194.9	5.7	2.09	30.56075	0.84	0.170
	5.56	227.9	3	2.35	41.71	1.13	0.140

	6.05	263.5	-0.2	3.2	28.2243	0.83	0.240
	6.91	330.4	9		462.24		-0.038
	7.86	391.9	8.8		100.8136		-0.033
	8.05	403.4	3.8		93.18644		0.033
	8.84	455.9	5.4				
	9.64	508.4			70.89831		-0.124
11	2.55	14.0	-0.58			insuff	insuff
	2.71	22.0	5.6	8.9	91.44	1.13	0.190
	3.05	36.0	0.6			1.58	0.280
	3.76	66.9	0.97			1.26	0.200
	5.29	135.8	6.1	5.8	44.12	1.04	0.190
	5.87	170.2	5	6.65	72.06	0.45	0.120
	7.05	235.8	0.56	4.24	61.35	0.38	0.270
	8.32	320.9	6.5	4.93	967	0.96	0.200
	9.16	370.8	3.2	5.09	74.6	1.15	0.320
	9.83	402.6	3.5	5.3	0.9	0.93	0.100
	11.00	483.8	6.6		82.9322		0.131
	11.98	551.3	5.2				
	12.23	573.8	3.9		56.83051		0.052
12	0.58	0.0	-0.95		190.0508		-0.128
	0.68	0.0	5.2		119.9661		-0.110
	0.79	0.0	2.6		109.5424		-0.113
	1.13	0.0	6.9				
	1.63	0.0			63.44068		-0.096
13	9.86	364.4	0.2	1.29	133.09	0.92	
	11.42	502.4	-0.08			0.55	0.190
	13.08	600.1	4.9	5.5	104.81	0.86	0.350
	15.92	740.1	3.48	1.9	83.01	0.60	0.190
	17.19	828.6	1.63	1.21	94.03	0.58	0.110
		70	3.9	1.41		1.66	0.080
			-0.5				0.016
			1.3				
14	4.26	48.9	3.14			0.88	insuff
	4.56	61.4	5.8	6.12	58.8	1.22	0.150
	5.81	130.5	4			1.37	insuff
	6.45	172.1	2.1	7.65		0.52	0.370
	7.23	229.5	5.2	4.38	33.83178	0.58	0.200
	8.04	281.8	5.47	4.29	91.67	0.18	-0.080
	8.87	349.1	4.44	5.16	79.9	0.77	0.110
	9.54	391.5	3	5.62	66.71	0.91	0.250
	10.23	432.0	1.3	5.49	26.4	0.89	0.100
	11.47	505.5	5.6		62.33898		-0.067
	12.22	554.5	4.5		80.30508		-0.062

	12.60	579.0	4.4		74.45763		-0.056
15	0.00	0.0	4.2				
	0.06	0.0	-1.1		107.43		
	0.54	0.0	1.73	2.84	108.99		0.680
	0.84	0.0	3.42	4.25	528.51		0.540
	0.93	0.0	-0.22	4.01	16.19		0.030
	1.03	0.0	2.46	3.47	235.41	0.43	0.000
	1.52	0.0	5.1	5.84	401.33	1.12	0.270
	1.98	0.0	5	5.24	290	0.85	0.220
	2.04	0.0		5.73	233	0.62	-0.130
	2.47		4	4.11		1.04	0.200
	2.79		4.9		140.48		0.087
	3.12	15.0	4.9		270.04		-0.047
	4.00	46.5	6.6		65.88		0.056
	4.73	72.0			84.81		0.088
16	0.00	0.0	-0.2	1.65	294.19	0.72	0.120
	0.00	0.0	-0.1		480.86	0.94	0.000
	0.04	0.0	1.2	7.23		0.67	0.230
	0.20	0.0	-0.35	3.71	174.9	0.67	0.110
	0.44	0.0	-1.23	6.68		1.27	0.040
	0.61	0.0	1.2		91.19		-0.027
	0.80	0.0	4.8				
	1.20	0.0	8.3		625.4		0.044
17	1.80	18.0	6.1	2.28	140.26	0.83	0.200
	2.28	40.4	1.35	3.4		1.22	0.280
	2.97	91.9	8.6			0.54	
	3.61	140.7	11.4	4.33	205.25	0.48	0.040
	4.16	176.7	5.4	2.21	57.65	0.59	0.000
	4.98	284.2	5.08	3.94	60.34	0.79	0.020
	5.38	341.4	4.9	5.05	32.9	0.84	0.080
	6.36	446.4		6.9	141.7757	0.52	0.100
	6.91	511.4	6.3		92.25424		-0.093
	7.47	566.4	6.6		62.42373		-0.047
	7.68	586.4	5.3		60.22034		-0.056
	8.58	665.4	9.2				
18	7.21	116.0	4.9	5.27	191.81	1.05	0.120
	7.80	376.0	2.5				
	8.88	438.0	1.27		272.1695		-0.074
	10.67	553.0					
	11.91	628.0			53.18644		-0.098
19	9.91	508.4	9.3		86.15254		0.029
	11.06	583.0			55.05085		-0.018
20	0.00	0.0	-0.34			0.91	insuff

	0.00	0.0	-0.6	17.88		0.73	0.13
	0.00	0.0	-0.14			1.23	0.16
	0.00	0.0	2.85			1.41	insuff
	0.00	0.0	0.7	19.8		0.72	0.27
	0.61	0.0	2.2	7.4	136.2	0.18	0.09
	0.99	0.0	-0.33	6.92	221.4	0.46	0.12
	1.21	0.0	3.29	7.06	302.6	0.72	0.11
	1.90	0.0	3.95	7.32	198.0	0.72	0.20
	3.07	35.0	1.8	8.01	273.9	1.03	0.24
	4.22	103.0	2.9	6.23		0.57	0.23
	5.29	160.7	2.6				
	5.55	175.6	0.43		119.2		0.00
	5.80	192.7	5.7		314.7		0.01
	6.31	227.7	7.2		312.3		-0.04
	7.75	340.4		12.4	161.9		-0.07
21	0.00	0.0	-0.1			1.17	insuff
	0.00	0.0	0.6	7.44		0.75	0.21
	0.00	0.0	0.42			0.99	0.17
	0.00	0.0	2.4				
	0.00	0.0	-0.1	112.75	271.4	0.76	0.24
	0.00	0.0	0.24	29.45	700.2	0.42	0.15
	0.00	0.0	-0.12	24.84	837.5	0.44	0.15
	0.00	0.0	-1.16	7.07	43.4	0.58	0.16
	0.62	0.0	5.52	5.56	91.8	0.57	0.11
	1.48	0.0	4.6	6.7	100.9	0.74	0.16
	2.96	0.0	4.7		49.3		0.19
	3.96	0.0	0.03		80.1		
	4.66	35.0	4.9		127.9		-0.04
	5.95	159.7		12.8	102.8		-0.11
22	0.00	0.0	-0.7	15.9	460.2	0.66	0.17
	0.00	0.0	2.8	23.5	813.7	0.47	0.22
	1.05	0.0	3	6.45	88.9	1.02	0.12
	3.97	0.0		11.8	56.2		-0.04
23	0.00	0.0	4.1	23.4	434.7	insuff	insuff
	0.00	0.0	3.6	21.15	452.4	0.22	0.09
	1.26	0.0	2.7	6.65	88.4	0.85	0.12
	1.50	0.0	5.1	17.2	24.0		-0.12
Mean	4.63	159.34	3.65	8.65	234.41	0.78	0.08
Median	3.74	47.68	3.98	5.73	98.44	0.73	0.09
SD	3.99	201.22	2.86	12.35	539.94	0.30	0.14
sem	0.28	14.34	0.20	0.88	38.47	0.02	0.01

	0.00	0.0	-0.6	17.88		0.73	0.13
	0.00	0.0	-0.14			1.23	0.16
	0.00	0.0	2.85			1.41	insuff
	0.00	0.0	0.7	19.8		0.72	0.27
	0.61	0.0	2.2	7.4	136.2	0.18	0.09
	0.99	0.0	-0.33	6.92	221.4	0.46	0.12
	1.21	0.0	3.29	7.06	302.6	0.72	0.11
	1.90	0.0	3.95	7.32	198.0	0.72	0.20
	3.07	35.0	1.8	8.01	273.9	1.03	0.24
	4.22	103.0	2.9	6.23		0.57	0.23
	5.29	160.7	2.6				
	5.55	175.6	0.43		119.2		0.00
	5.80	192.7	5.7		314.7		0.01
	6.31	227.7	7.2		312.3		-0.04
	7.75	340.4		12.4	161.9		-0.07
21	0.00	0.0	-0.1			1.17	insuff
	0.00	0.0	0.6	7.44		0.75	0.21
	0.00	0.0	0.42			0.99	0.17
	0.00	0.0	2.4				
	0.00	0.0	-0.1	112.75	271.4	0.76	0.24
	0.00	0.0	0.24	29.45	700.2	0.42	0.15
	0.00	0.0	-0.12	24.84	837.5	0.44	0.15
	0.00	0.0	-1.16	7.07	43.4	0.58	0.16
	0.62	0.0	5.52	5.56	91.8	0.57	0.11
	1.48	0.0	4.6	6.7	100.9	0.74	0.16
	2.96	0.0	4.7		49.3		0.19
	3.96	0.0	0.03		80.1		
	4.66	35.0	4.9		127.9		-0.04
	5.95	159.7		12.8	102.8		-0.11
22	0.00	0.0	-0.7	15.9	460.2	0.66	0.17
	0.00	0.0	2.8	23.5	813.7	0.47	0.22
	1.05	0.0	3	6.45	88.9	1.02	0.12
	3.97	0.0		11.8	56.2		-0.04
23	0.00	0.0	4.1	23.4	434.7	insuff	insuff
	0.00	0.0	3.6	21.15	452.4	0.22	0.09
	1.26	0.0	2.7	6.65	88.4	0.85	0.12
	1.50	0.0	5.1	17.2	24.0		-0.12
Mean	4.63	159.34	3.65	8.65	234.41	0.78	0.08
Median	3.74	47.68	3.98	5.73	98.44	0.73	0.09
SD	3.99	201.22	2.86	12.35	539.94	0.30	0.14
sem	0.28	14.34	0.20	0.88	38.47	0.02	0.01

All NTCK patients

	Iron load	dfo grams	NTBPI	Epo	Sol transferrin rcptr	mda	carbonyls
1	1.66	95.0	7.81	2758.4	7.97	0.03	0.25
	3.34	196.0	4.90			0.52	1.56
	3.80	214.0	1.30			0.00	0.74
2	1.90	30.0	6.99	501.3	15.52	0.23	0.48
3	1.07	11.5	8.36	461.3	5.13	0.14	1.92
	2.64	59.0	3.80	410.9		0.39	0.89
4	1.07	24.0	6.31	524.2	6.68	0.29	0.19
	2.49	91.5		1229.4			
	2.94		1.33			0.28	0.59
	4.31						
5	0.71	0.0	6.82	1007.0		0.25	0.05
	1.07	24.0	1.50	415.4	4.29	0.07	0.58
	1.67		2.70	243.3		0.51	0.94
	2.08		0.69	25.4		0.23	0.70
6	1.16	0.0	2.13	5705.4	23.44	0.22	0.25
	1.66	0.0	-0.61	638.3	21.92	0.03	0.39
	2.53	4.5	2.60	4369.0		0.44	0.63
	2.98						
7	0.25		8.65	4346.5	25.24	0.36	0.31
	0.31		4.54	618.8	14.92	0.03	0.92
	0.49		3.40	220.9		0.55	1.18
	0.62		1.26	131.4		0.55	0.95
8	2.98	42.5	12.16	486.9	8.07	0.06	0.51
	2.57		7.40	605.1		1.84	0.94
9	0.81		10.04	531.5	8.09	0.21	
10	0.41		1.61	3680.8	32	0.05	
	0.71		-1.00	1633.2	14.32	-0.02	0.27
	1.59		4.70	693.4	8.3	0.36	1.07
	2.25	2.0					
	10.61	239.0					
11	0.41		1.71	507.2	14.2	0.26	
	0.41		0.30	702.3			
12	1.32	36.5	-0.25	44.2		0.26	1.01
13	0.20		4.86	547.2		0.10	0.10
	0.60			1018.5		0.21	0.77
	0.76		0.87			0.00	0.90
14	2.15	93.0	6.80	5045.6	19.92	0.25	0.13
	3.22	115.5	0.65	118.8	3.02	0.07	0.59
	4.13	137.5	3.50	979.0		0.59	1.30

	5.25	169.0	1.52	170.7		0.48	0.70
	7.18	234.5	6.30				
15	0.32		6.81	459.3	5.83	0.22	0.36
	1.04		3.20	51.9			0.84
	1.44	8.0	1.93	95.9		0.46	0.66
16	4.02	167.5	3.68	491.6	4.07	0.21	0.59
	6.66	295.5	1.82			0.35	0.63
17	3.76	159.5	1.39	-			
	5.43	268.0	3.70	44.1		0.53	1.18
	5.89	305.5	3.91	2254.9		0.26	0.47
18	5.85	217.0	2.69	-			
	7.07	367.0	2.60	238.0		0.77	1.36
	7.07	385.0	4.06				
19	0.36		1.61	820.7	13.76	0.20	0.75
	0.91		3.30	18.2		0.33	1.22
20	3.25	36.5		1653.6		2.06	
21	1.83	91.0	7.40	2703.9		1.19	
22	0.20		0.62		21.72	0.29	0.99
23	0.00	-		96.9		0.83	
24	1.27		5.40	146.4		0.41	
25	1.12		-0.32	19.3		0.53	
26	1.83		4.60	3466.5		1.57	
27	0.00		0.14	282.5			
Mean	2.38	124.8	3.596116	1108.646	13.25762	0.40	0.734803
Median	1.67	93.0	3.25	515.73	13.76	0.27	0.696865
SD	2.22	115.0	2.929614	1445.848	8.185374	0.43	0.409514
SEM	0.27	14.2	0.360611	177.9715	1.00755	0.05	0.050408